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Attorney's Docket No. 035718/237005(5718-118)

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In The United States Patent And Trademark Office

Appl. No.: 10/032,717

Confirmation No.: 5409

Applicant(s): Abad et al.

Filed: October 23, 2001

Art Unit: 1638

Examiner: Anne R. Kubelik

Title: GENES ENCODING NOVEL BACILLUS THURINGIENSIS PROTEINS
WITH PESTICIDAL ACTIVITY AGAINST COLEOPTERANS

Docket No.: 035718/237005 (5718-118)

Customer No.: 29122

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450APPEAL BRIEF TRANSMITTAL
(PATENT APPLICATION - 37 C.F.R. § 41.37)

1. Transmitted herewith is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on July 6, 2005.
2. ☐ Applicant claims small entity status.
3. Pursuant to 37 C.F.R. § 41.20(b)(2), the fee for filing the Appeal Brief is:
☐ small entity \$250.00
☒ other than small entity \$500.00

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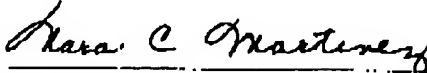
- ☒ Please charge the \$500.00 Appeal Brief Fee and any additional fee or refund to Deposit Account 16-0605.

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Date

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APPEAL BRIEF UNDER 37 CFR § 41.37

This Appeal Brief is filed pursuant to the "Notice of Appeal to the Board of Patent Appeals and Interferences" filed July 6, 2005 and the Amendment After Final submitted herewith. Applicants respectfully request that this Appeal Brief and the Amendment be entered in the case.

1. ***Real Party in Interest.***

The real party in interest in this appeal is E.I. du Pont de Nemours and Company, the assignee of the above-referenced patent application.

2. ***Related Appeals and Interferences.***

There are no related appeals and/or interferences involving this application or its subject matter.

3. ***Status of Claims.***

Claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 have been rejected and are the subject of this appeal. Claims 39, 40, 44, 45, 50, and 51 have been objected to but would be allowable if rewritten in independent form including all of the limitations of the base claim and

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any intervening claims (Office Action of 5/6/05, page 13, #7). Claims 4-8, 13-16, 20-27, 41, 42, 47, 48, 53, 54, and 65 have been cancelled.

4. Status of Amendments.

Applicants are filing herewith an Amendment After Final in order to cancel claim 65. With the entry of this Amendment After Final, the number of issues on appeal will be reduced to two issues as set forth below.

5. Summary of Claimed Subject Matter.

The pending claims of the present invention are drawn to an isolated nucleic acid comprising a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1, wherein said nucleotide sequence encodes a polypeptide which is pesticidal for at least one pest belonging to the order Coleoptera (see, e.g., page 11, line 18; page 12, line 10; page 18, line 29; and page 3, line 20). The claims of the present invention are also drawn to a transformed plant comprising in its genome at least one stably incorporated nucleotide construct comprising a nucleotide sequence encoding a polypeptide operably linked to a promoter that drives expression of said polypeptide, wherein said polypeptide is pesticidal for at least one pest belonging to the order Coleoptera and wherein said nucleotide sequence has at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1 (see, e.g., page and line numbers cited above as well as page 7, line 5). The claims of the present invention are also drawn to a method for impacting an insect pest comprising introducing into a plant or cell thereof at least one nucleotide construct comprising a nucleotide sequence encoding a polypeptide operably linked to a promoter that drives expression of said polypeptide in plant cells, wherein said polypeptide is pesticidal for at least one pest belonging to the order Coleoptera and wherein said nucleotide sequence has at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1, whereby an insect pest feeding on said plant or cell thereof is impacted (see, e.g., page and line numbers cited above as well as page 7, line 6).

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6. *Grounds of Rejection to be Reviewed on Appeal.*

Issue 1—Whether claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

Issue 2—Whether claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 meet the written description requirement of 35 U.S.C. § 112, first paragraph.

7. *Grouping of Claims.*

Applicants believe that the claims do not stand or fall together. The rejected claims are all "sequence identity claims" which contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1. However, some of the sequence identity claims differ from each other in the minimum percent sequence identity that is required and may therefore be found to differ in meeting the requirements of patentability. That is, claims 1, 9, and 17 require that the nucleotide sequence has at least 90% sequence identity to SEQ ID NO:1, while claims 55, 58, and 63 contain limitations requiring the nucleotide sequence to have at least 93% identity to SEQ ID NO:1. Claims 56, 59, and 64 contain limitations requiring the nucleotide sequence to have at least 94% identity to SEQ ID NO:1, and claims 38, 43, and 49 contain limitations requiring the nucleotide sequence to have at least 95% identity to SEQ ID NO:1. While Applicants believe that all these claims are allowable, it is conceivable that among claims with differing requirements for percent sequence identity, some claims could be found to meet the enablement and written description requirements while others might not. Therefore, the sequence identity claims do not necessarily all stand or fall together. For example, claims requiring at least 93% sequence identity may stand or fall separately from those claims requiring at least 95% sequence identity.

8. *Argument.*

(a) Issue 1—Whether claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

In the final Office Action (5/6/05, page 2, #4), the Examiner rejected claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-65 under 35 U.S.C. § 112, first paragraph, because:

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the specification, while being enabling for nucleic acids encoding SEQ ID NO:2 and 10, expression cassettes comprising the nucleic acids, plants and seeds comprising a construct comprising the nucleic acid and a method of using it to impact a plant pest, does not reasonably provide enablement for any nucleic acid that has 90% identity to SEQ ID NO:1, expression cassettes comprising the nucleic acid, plants and seeds comprising a construct comprising the nucleic acid and a method of using it to impact a plant pest.

Applicants are submitting herewith an Amendment After Final to cancel claim 65. Accordingly, this rejection will be discussed with regard to the remaining sequence identity claims.

The enablement rejection encompasses claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64. These claims contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1 and thus are referred to herein as the "sequence identity claims." Applicants respectfully traverse this rejection and submit that the Examiner is applying an extraordinarily high standard of enablement to the present claims, a standard that is not properly based on case law or on the statute.

Applicants note that the arguments contained herein are similar to those in the Appeal Brief on the same issues filed in this case on 6/1/04 in response to the final Office Action of 12/3/03. After that Appeal Brief was filed, the Examiner reopened prosecution after making an additional rejection under 35 U.S.C. §103. That rejection under 35 U.S.C. §103 has since been withdrawn (see Office Action of 5/6/05, page 2, #3), but the rejections under 35 U.S.C. §112 were similar to those made earlier and are addressed by the following arguments.

Support is provided for the limitations of the claims

First, guidance is provided as to what sequence alterations may be made and still provide a pesticidal polypeptide encompassed by the claim. As discussed further below, endotoxin genes are well known in the art. Applicants have provided the exemplary nucleotide sequence of SEQ ID NO:1 and the exemplary amino acid sequence of SEQ ID NO:2. Indeed, the Office Action states that the specification is enabling for nucleic acids encoding SEQ ID NO:2 (Office Action of 5/6/05, p. 2, #4, first paragraph), and the Examiner indicated that claims drawn to the exemplary disclosed sequences (*i.e.*, claims 39, 40, 44, 45, 50, and 51) would be allowable if

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rewritten in independent form (Office Action of 5/6/05, p. 13, #7). Applicants further note that the Office Action (5/6/05, page 8, last paragraph) states that "[p]lant transformation and construct [*sic*] of DNA constructs, per se, are enabled."

The claimed sequences of the invention vary from the exemplary disclosed sequences by structural parameters (*i.e.*, percent sequence identity to SEQ ID NO:1; encoding the amino acid sequence set forth in SEQ ID NO:2). Guidance for determining percent identity of sequences is provided in the specification on pages 33 through 38. Moreover, the independent sequence identity claims (*i.e.*, claims 1, 9, and 17) specify that the nucleotide sequence encodes a polypeptide which is pesticidal for at least one pest belonging to the order Coleoptera; therefore, these claims (and the claims dependent on them) encompass functional variants. Guidance regarding alterations that allow the sequence to retain the specified pesticidal activity is also provided (see, *e.g.*, p. 18 (providing guidance regarding conservative substitutions of amino acids) and pp. 19-20 (discussing the activity of variants)).

Methods for assaying the pesticidal activity of proteins are routine in the art and are also described in the specification, for example, on pages 8 and 29 and in the experimental section in working examples such as Example 4 (pp. 65-66), Example 6 (p. 67), and Example 7 (p. 69). These working examples teach methods for assaying pesticidal activity of proteins and demonstrate results obtained using these assays. In this manner, Applicants have provided guidance regarding what changes may be made to allow the endotoxin sequence to retain the specified pesticidal activity.

B. thuringiensis δ -endotoxins are well-known in the art, and further support and guidance is provided by working examples

The Examiner concluded in the Advisory Action of 2/18/04 that "[t]he specification provides no guidance as to which amino acids of SEQ ID NO:2 are critical for function." In the Office Action of 5/6/05 (page 4, third full paragraph), the Examiner concludes that "[t]he specification does not teach how to make nucleic acids encoding Coleopteran pesticidal proteins

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with 181, 217, 253 or 326 substitutions." Applicants respectfully disagree with these conclusions¹.

As noted above, Applicants have provided the exemplary nucleotide sequence of SEQ ID NO:1 and the exemplary amino acid sequence of SEQ ID NO:2. The claimed sequences of the invention vary from this sequence by structural parameters (*i.e.*, percent sequence identity to SEQ ID NO:1). As discussed extensively in the specification (*e.g.*, pp. 3, 7, 11-12, 15, 24-25), the disclosed exemplary sequence of SEQ ID NO:2 is a *Bacillus thuringiensis* Cry-8-like δ -endotoxin. The *B. thuringiensis* δ -endotoxins are an extremely well-characterized group of proteins. As discussed in the specification at pp. 24-25:

Many of the δ -endotoxins are related to various degrees by similarities in their amino acid sequences and tertiary structure, and means for obtaining the crystal structures of *B. thuringiensis* endotoxins are well known. Exemplary high-resolution crystal structure solution of both the Cry3A and Cry3B polypeptides are available in the literature. The inventors of the present invention used the solved structure of the *Cry3A* gene (Li et al. (1991) *Nature* 353: 815-821²) to produce a homology model of the Cry8 δ -endotoxin disclosed and claimed herein as SEQ ID NO:2 to gain insight into the relationship between structure and function of the endotoxin, and to design the recombinantly engineered proteins disclosed and claimed herein. A combined consideration of the published structural analyses of *B. thuringiensis* endotoxins and the reported function associated with particular structures, motifs, and the like indicates that specific regions of the endotoxin are correlated with particular functions and discrete steps of the mode of action of the protein. For example, δ -endotoxins isolated from *B. thuringiensis* are generally described as comprising three domains, a seven-helix bundle that is involved in pore formation, a three-sheet domain that has been implicated in receptor binding, and a beta-sandwich motif (Li et al. (1991) *Nature*, 305: 815-821).²

¹ Applicants note that the Examiner refers throughout the Office Action to various numbers of nucleotide changes (*e.g.*, page 3, paragraph 4), almost as if the claims required that these numbers of nucleotide substitutions be made. Applicants wish to emphasize that the claims pending in the case contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1 and do not actually require the modification of particular numbers of nucleotides, as the Examiner seems to suggest throughout the Office Action.

² Submitted herewith as Appendix A; submitted with the Amendment of 1/21/05 as Appendix A.

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As discussed in more detail in the specification (see, e.g., p. 25), the inventors made use of this knowledge in the art to design specific mutations in the Cry8-like proteins to enhance their pesticidal activity. This strategy was successful in creating altered endotoxins with increased toxicity, as demonstrated by the data presented in working Example 6 (pp. 67-69). Thus, as demonstrated by working examples in the specification, those of skill in the art (i.e., the inventors) were able, in view of the extensive knowledge in the art about *B. thuringiensis* δ -endotoxin structure and function, to modify the exemplary wildtype sequences disclosed herein to provide variant endotoxins with enhanced pesticidal activity. In this manner, the data provided in the specification (e.g., Example 6) demonstrate that one of skill in the art would know what amino acids could be changed to provide a protein with pesticidal activity. Accordingly, Applicants submit that adequate guidance is provided as to which amino acids of SEQ ID NO:2 are critical for function.

The data and working examples provided in the specification also demonstrate the enablement of the claimed invention by showing that sequences of the invention that share a relatively low percent identity to the exemplary sequence of SEQ ID NO:1 encode polypeptides that have pesticidal activity against several Coleopteran pests. In Example 4 (specification pp. 65-66), both the full-length endotoxin encoded by SEQ ID NO:1 and a truncated protein encoded by SEQ ID NO:15 were assayed for pesticidal activity against southern corn rootworm. The nucleotide sequence of SEQ ID NO:15 is a truncation of SEQ ID NO:1 which shares about 55% sequence identity with SEQ ID NO:1. In Example 6 (specification pp. 67-69), several truncated proteins were assayed and shown to have pesticidal activity against Colorado potato beetle (see Table 1, p. 68). These truncated proteins included those encoded by SEQ ID NO:15 and SEQ ID NO:19, which share about 55% and 51% identity, respectively, to the exemplary nucleic acid sequence set forth in SEQ ID NO:1 (alignments performed using BLAST with default parameters).

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As briefly discussed above, Example 6 also provides assay data for a mutated sequence, NGSR1218-1. This NGSR1218-1 mutant includes the amino acid sequence "NGSR" inserted between amino acids 164 and 165 of the truncated endotoxin of SEQ ID NO:16. The nucleotide sequence encoding this mutant (SEQ ID NO:11) shares about 56% sequence identity with the exemplary nucleotide sequence of SEQ ID NO:1, yet as documented by the data provided in Example 6, both proteins have pesticidal activity. In addition to this data, the specification also provides an exemplary maize-optimized sequence (SEQ ID NO:9) which encodes the same pesticidal polypeptide as SEQ ID NO:15 but shares less than 69% sequence identity with it. Thus, the specification is replete with working examples of sequences that share a relatively low percentage of identity with SEQ ID NO:1 and which encode polypeptides having pesticidal activity. In fact, the percentage of sequence identity shared by the exemplary SEQ ID NO:1 and these sequences in the working examples is much lower than the "at least 90%" of the broadest sequence identity claims.

The Examiner previously dismissed the working examples provided by Applicants, concluding that the specification teaches only "a fragment," "a single insertion of 4 amino acids in the 669 amino acid long SEQ ID NO:16," and "nucleic acids encoding SEQ ID NO:2" and stating that the specification "is not enabled for nucleic acids that have 90% identity to SEQ ID NO:1 but that do not encode SEQ ID NO:2." (Advisory Action of 2/18/04) The Examiner now dismisses the working examples provided by Applicants, stating that "the percent identity variants taught by the specification do not represent the full scope of the claims" and that "[n]one of the working examples in the specification modified 181, 217, 253, or 362 amino acids" (Office Action of 5/6/05, page 7, second paragraph through fourth paragraph).

Applicants respectfully disagree with this analysis and these conclusions. Contrary to the Examiner's assertion, it is not necessary to make and use every embodiment within the scope of the claims in order for the claims to be enabled. Rather, what is required for enablement is that a disclosure must contain sufficient information to enable one skilled in the pertinent art to make and use the claimed invention (MPEP § 2164.01). Applicants have provided the exemplary

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wildtype sequences of SEQ ID NO:1 and the encoded SEQ ID NO:2 and further have provided working examples of percent identity variants including both fragments and amino acid changes to these exemplary sequences; thus, Applicants have taught representative species of the genus of sequences having a particular structural relationship to the exemplary wildtype sequences. In view of these teachings, Applicants respectfully submit that the Examiner has not met her burden of establishing a reasonable basis to question the enablement provided for the claimed invention (MPEP § 2164.04).

The amount of experimentation required to make and use the subject matter of the claims is not undue

The Examiner concludes that "the amount of experimentation required would be undue," reasoning as follows:

[M]aking all possible single amino acid substitutions in an 3621 nucleotide long nucleic acid like that of SEQ ID NO:1 would require making and analyzing 19^{3621} nucleic acids; these nucleic acids would have about 99.99% identity to SEQ ID NO:1. Making the nucleic acids this way would be required because of the lack of guidance provided in the specification. Thus, in the instant case, the amount of experimentation required would be undue.

(Office Action of 5/6/05, page 10, second full paragraph)

Applicants respectfully disagree with this analysis and conclusion. Contrary to the Examiner's assertion, it is not necessary to make and use every embodiment within the scope of the claims in order for the claims to be enabled. Rather, what is required for enablement is that a disclosure must contain sufficient information to enable one skilled in the pertinent art to make and use the claimed invention (MPEP § 2164.01).

The Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation needed to practice the invention is not undue, and that a considerable amount of experimentation is permissible if it is merely routine or if the specification provides a reasonable amount of guidance as to how the experimentation should proceed. *Id. In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed Cir 1988). In the instant case, the quantity of experimentation required to practice independent

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claim 1 amounts to two steps: (1) generating a nucleic acid comprising a nucleotide sequence that has at least 90% sequence identity to SEQ ID NO:1; and (2) assaying the encoded polypeptide for functional activity. Such assays, while known in the art, have further been presented in the specification. One of skill in the art would appreciate that both of these steps are within the skill of those in the art and that this degree of experimentation is not considered undue.

Similarly, the amount of experimentation needed to practice the other sequence identity claims is not undue. For example, independent claim 9 recites a transformed plant comprising a nucleotide construct that has a nucleotide sequence with at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1 and that encodes a polypeptide that is pesticidal for at least one pest belonging to the order Coleoptera. Thus, in addition to the steps required to practice independent claim 1, independent claim 9 requires the transformation of a plant. Plant transformation is routine in the art; thus, the amount of experimentation required to practice claim 9 is not undue. Similarly, in addition to the steps required to practice independent claim 1, the method of independent claim 17 requires that a nucleotide construct be created in which the nucleotide sequence is operably linked to a promoter; that the construct be introduced into a plant or cell thereof; and that an insect pest feeding on said plant or cell is impacted. The performance and/or evaluation required by each of these additional steps is within the skill of those in the art and would not be considered undue experimentation by those in the art. Likewise, the remaining sequence identity claims, which are all dependent on or incorporate the limitations of independent claim 1, 9, or 17, contain additional requirements which are equally within the skill of those in the art.

Applicants note that it is now customary in the art to make and assay a number of sequences for a desired function in order to achieve the best results. For example, common techniques involve what is often referred to as "shuffling," as described for example in U.S. Patent No. 5,837,458, issued November 17, 1998 with inventors Minshull and Stemmer and entitled, "Methods and Compositions for Metabolic and Cellular Engineering." With such techniques, it is common to mutagenize individual sequences or a set of sequences which are

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then assayed for a desired activity. Such techniques may even make use of a library of sequences which is recursively mutagenized, screened for function using a functional assay, and re-mutagenized in order to find a sequence exhibiting optimal function. Examples of the use of such techniques include: Minshull and Stemmer (1999) *Current Opinion in Chemical Biology* 3:284-290, entitled "Protein Evolution by Molecular Breeding"; and Christians *et al.* (1999) *Nature Biotechnology* 17: 259-264, entitled "Directed evolution of thymidine kinase for AZT phosphorylation using DNA family shuffling." Such experiments are designed and are intended to encompass the generation and testing of a very large number of variant sequences for a desired function. As indicated by these and other publications in the art, this level of experimentation is now considered routine in the art and thus would not be considered "undue experimentation" under *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed Cir 1988) and *In re Jackson*, 217 USPQ 804, 807 (Bd. Pat. App. & Int. 1982) (holding that a considerable amount of experimentation is permitted to practice the invention and is not undue if it is merely routine in the art or if the specification provides a reasonable amount of guidance and direction to perform such experimentation).

Further, Applicants note that Dr. André Abad submitted a Rule 132 declaration with the Amendment of 1/21/05³ stating, among other things that the procedures described in Examples 4, 6, and 7 of the specification are considered "routine." The Examiner has dismissed Dr. Abad's declaration as "only provid[ing] opinions, and ones the Declarant is not even sure of" (Office Action of 5/6/05, page 8, second and third paragraphs). Applicants believe that this summary by the Examiner is inaccurate and improper. Applicants maintain that present claims meet the enablement standard and believe that the Examiner has not met her burden of showing that the practice of the claims would require undue experimentation.

One of skill in the art could make and use the claimed invention without undue experimentation

It is true that some embodiments of the nucleotide sequence which meet the percent identity limitation of the claims may not encode a polypeptide that has the specified pesticidal activity. However, one of skill would readily be able to use the assays taught in the specification

³ Submitted herewith as Appendix C; submitted with the Amendment of 1/21/05 as Appendix C.

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to determine which nucleotide sequences that met the sequence identity limitations of the claims also encoded polypeptides having the specified pesticidal activity. Applicants note that the presence of inoperative embodiments within the scope of the claims does not render the claims invalid. *Atlas Powder Co. v. E.I. duPont de Nemours & Co.*, 750 F.2d 1569, 224 USPQ 409 (Fed. Cir. 1984). Nor would the amount of experimentation required to test a particular polypeptide for pesticidal activity be considered undue by one of skill in the art, as evidenced by the assay results presented in the specification, for example, in working Examples 4 (pp. 65-66), 6 (p. 67), and 7 (p. 69). The references cited by the Examiner—Lazar *et al.* (1988) *Mol. Cell. Biol.* 8: 1247-52 and Hill *et al.* (1998) *Biochem. Biophys. Res. Comm.* 244: 573-577—illustrate that one of skill would readily be able to determine whether a particular sequence change affected the function of a protein. Accordingly, one of skill in the art would be able to determine the functionality of polypeptides encompassed by the claimed invention without undue experimentation.

The Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation needed to practice the invention is not undue. *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed Cir 1988). Furthermore, a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance in which the experimentation should proceed. *Id.* Applicants stress that when evaluating the quantity of experimentation required, the court looks to the amount of experimentation required to practice a single embodiment of the invention rather than the amount required to practice every embodiment of the invention as the Examiner implies. For example, in *Wands*, the claims at issue were drawn to immunoassay methods using any monoclonal antibody having a binding affinity for HbsAg of at least 10^{-9} M. The PTO had taken the position that the claim was not enabled because it would take undue experimentation to make the monoclonal antibodies required for the assay. The Federal Circuit reversed and held that the claims were enabled, as the amount of experimentation required to isolate monoclonal antibodies and screen for those having the correct affinity was not undue. *See Id.* Clearly, the Federal Circuit did not contemplate that every antibody useful in the methods of the claim must be identified. Rather, the court considered the amount of experimentation required to identify one or a few monoclonal antibodies having the required

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affinity. *See also, Johns Hopkins University v. Cellpro*, 931 F. Supp. 303, 324 (D. Del. 1996), *aff'd in part, vacated in part, and remanded*, 47 USPQ2d 1705 (Fed. Cir. 1998) (stating that "[t]he specification need only enable one mode of making the claimed invention.").

Thus, for the reasons discussed above, Applicants respectfully submit that the sequence identity claims meet the enablement requirement of 35 U.S.C. §112, first paragraph. Applicants have provided the exemplary nucleotide sequence of SEQ ID NO:1 and the exemplary amino acid sequence of SEQ ID NO:2. Indeed, the Office Action states that the specification is enabling for nucleic acids encoding SEQ ID NO:2 (Office Action of 5/6/05, p. 2, #4, first paragraph), and the Examiner indicated that claims drawn to the exemplary disclosed sequences (*i.e.*, claims 39, 40, 44, 45, 50, and 51) would be allowable if rewritten in independent form (Office Action of 5/6/05, p. 13, #7). Based on the knowledge in the art and the guidance provided in the specification, the skilled artisan could choose among possible sequence modifications to produce polypeptides within the sequence identity parameters set forth in the claims and then test these sequence variants to determine if they retained pesticidal activity. The amount of experimentation needed to perform such an evaluation would not be considered by those of skill in the art to be undue; therefore, the amount of guidance presented in the specification is sufficient to enable the claims. Accordingly, Applicants respectfully submit that the Examiner has not met her burden of establishing a reasonable basis to question the enablement provided for the claimed invention (MPEP § 2164.04) and therefore Applicants respectfully submit that the Examiner's rejection of the sequence identity claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 under 35 U.S.C. § 112, first paragraph, for lack of enablement should be reversed.

Responses to Miscellaneous Statements in the Final Office Action

The Office Action of 5/6/05 contained numerous statements by the Examiner which seemed less relevant to a determination of enablement than those discussed above. Nevertheless, in order to be responsive to the Office Action, Applicants here address those statements in the Office Action that were in the portion of the Office Action directed to the enablement rejection and that were not addressed earlier in this brief.

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On page 3 of the Office Action of 5/6/05 (first and second paragraphs), the Examiner states that she is not persuaded by Applicants' statements that claims limited to only the exact exemplary sequences would not provide Applicants with any meaningful patent protection. The Examiner explains her reasoning as follows:

The claims are not drawn to modification of only a few positions, but to modification of up to 362 nucleotides or amino acids. If, as Applicant contends, it is 'relatively simple' to modify 362 nucleotides, then it would also be 'relatively simple' to modify 363 nucleotides, thus avoiding the scope of the claim. Thus, applicant's argument about teaching the public to make and use the invention without obtaining any protection it *[sic]* contradictory to Applicant's argument that nucleic acids with 90% identity to SEQ ID NO:1 are enabled.

Applicants are not sure they understand this statement, but Applicants disagree with both the Examiner's apparent reasoning and her stated conclusion. The Examiner appears to be arguing that because Applicants are willing to accept some limitations on the scope of their claims (*i.e.*, a nucleotide sequence having at least 90% sequence identity to SEQ ID NO:1), it necessarily follows that Applicants' arguments that extremely narrow claims offer no meaningful patent protection are without merit. Moreover, the Examiner appears to be arguing that somehow Applicants' statements regarding enablement are contradicted by their willingness to accept some limitations on the scope of their claims. Applicants are baffled by this apparent reasoning, because none of these conclusions seem to follow logically from the statements made by the Examiner. Indeed, given the state of the art (see also Rule 132 affidavit of Dr. André Abad, submitted with Amendment of 1/21/05⁴), it seems likely that even claims specifying much lower percentages of sequence identity could also be circumvented fairly easily; however, the Patent Office has been unwilling to allow claims that specify very low percentages of sequence identity. Applicants do not see any conflict between seeking claims that the Patent Office has been willing to allow in the past (*i.e.*, claims specifying at least 90% sequence identity to a particular sequence) and maintaining that claims limited to a particular sequence could be easily circumvented by a would-be infringer.

⁴ Submitted herewith as Appendix C; submitted with the Amendment of 1/21/05 as Appendix C.

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For clarity, Applicants' original statement regarding this issue is reproduced below as it appeared in Applicants' Amendment of 1/21/05 (pages 7-8):

The rejected claims contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1 and thus are referred to herein as "sequence identity claims." The crux of the disagreement about the rejections of the sequence identity claims is that Applicants believe it is unreasonable for the Office Actions to seek to limit the claims to only the exact exemplary sequences disclosed in the specification, because as is well known to those of skill in the art, it is a relatively simple task to modify a nucleotide sequence and corresponding amino acid sequence at a few positions to generate a protein that retains the full activity of the original—that is, it is easy for one of skill in the art to essentially duplicate the claimed invention using a slightly modified sequence. However, such a sequence would fall outside the literal scope of a claim that was drawn only to the original sequence. Thus, if the claims are limited to the exact exemplary sequence disclosed as suggested in the Office Action (10/21/2004, page 2, #4), Applicants will in fact have taught the public how to make and use the invention without obtaining any meaningful protection which provides the incentive to invention contemplated in the Constitution (Article I, Section 8, clause 8, which gives Congress the power "[t]o promote the Progress of Science and useful Arts, by securing for limited times to Authors and Inventors the exclusive Right to their respective Writings and Discoveries.") Accordingly, Applicants respectfully submit that sequence identity claims should be viewed favorably and that the pending claims should be allowed.

On page 3 of the Office Action of 5/6/05 (third and fourth paragraphs), the Examiner states that Applicants' statements were not persuasive where Applicants stated that claims specifying at least 93%, 94%, and 95% sequence identity were ignored. Applicants note that the enablement rejection in the Office Action of 12/3/03 which was the subject of the first appeal in this case did indeed ignore these claim limitations, and Applicants raised the point in their subsequent response to ensure that these claims were considered separately in case they would be found to be enabled where claims specifying lower percentages of sequence identity were not.

In this regard, Applicants also wish to clarify another issue raised by the Examiner. Applicants note that the Examiner refers throughout the Office Action to various numbers of nucleotide changes (e.g., page 3, paragraph 4), almost as if the claims required that these

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numbers of nucleotide substitutions be made. Applicants wish to emphasize that the claims pending in the case contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1, *i.e.*, at least 90% sequence identity. The claims do not, as the Examiner seems to suggest, require the modification of particular numbers of nucleotides (*sec, e.g.*, page 3, fourth paragraph; page 4, second paragraph and fourth paragraph; paragraph bridging pages 4-5; page 5, second full paragraph; page 6, first full paragraph; page 6, last paragraph; page 7, fourth paragraph; page 8, first and last paragraphs).

On page 5 of the Office Action of 5/6/05 (third and fourth full paragraphs), the Examiner states that Applicants' assertions that those of skill in the art are aware of conserved regions of the Cry endotoxins are unpersuasive. For clarity, Applicants' original statement regarding the Pfam alignments submitted with the Amendment of 1/21/05 (page 12) is reproduced below:

In addition to the tertiary structure of an exemplary Cry endotoxin taught by the Li reference, those of skill in the art are aware of conserved regions of the Cry endotoxins. Provided herewith as Appendix B is an alignment of exemplary SEQ ID NO:2 with the Pfam consensus domains for endotoxins, which are referred to as "Endotoxin N," "Endotoxin M," and "Endotoxin C" (Pfam accession numbers PF03945, PF00555, and PF03944, respectively, descriptions of which are also attached as Appendix B). The Pfam database provides a curated collection of well-characterized protein family domains with high quality alignments. It is well known in the art that regions of sequence homology with known functional domains may be used to determine protein function and to identify what regions of a protein are particularly conserved (and therefore less likely to tolerate mutations) as well as what regions of a protein are less conserved (and therefore more likely to tolerate mutations). Accordingly, Applicants submit that the novel exemplary sequences disclosed in the specification, combined with the knowledge of one familiar with the art, provide adequate guidance as to which amino acids of SEQ ID NO:2 may be modified while allowing the protein to retain pesticidal activity.

The Pfam alignments submitted with the Amendment of 1/21/05⁵ include detailed domain descriptions for each of "Endotoxin N," "Endotoxin M," and "Endotoxin C"; each of

⁵ Submitted herewith as Appendix B; submitted with the Amendment of 1/21/05 as Appendix B.

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these descriptions cites the Li reference⁶ (Li *et al.* (1991) *Nature* 353: 815-821) discussed in the specification and discussed herein at length earlier as well as Schnepf *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62: 775-806, indicating that the critical information regarding these domains was known in the art well before the present application was filed.

Moreover, the very passage of the 1991 Li reference cited by the Examiner (Office Action of 5/6/05, page 5, fourth full paragraph) emphasizes Applicants' point regarding the extensive knowledge in the art regarding Cry endotoxins. This passage (Li reference p. 815, column I) is as follows:

The δ -endotoxins are a family of insecticidal proteins produced by *Bacillus thuringiensis* (B.t.) during sporulation, having relative molecular masses (M_r) 60,000-70,000 (60K-70K) in the active form and specific toxicities against insects in the orders of Lepidoptera, Diptera, and Coleoptera. These toxins have been formulated into commercial insecticides for three decades, and now insect-resistant plants are engineered by transformation with Lepidoptera-specific toxin genes. In the bacterium δ -endotoxins are synthesized as protoxins of M_r s 70K-135K and crystallize as a parasporal inclusion $\sim 1\mu$ in size, in which form they are ingested by the susceptible insect. The microcrystal dissolves in the alkaline pH of the midgut and the protoxin is cleaved by gut proteases to release the active toxin. δ -Endotoxins activated *in vitro* bind specifically and with high affinity ($K_D \approx 0.1$ -20 nM) to protein receptors on brush-border membrane vesicles derived from the gut epithelium of target insects and create leakage channels of 10-20 Å diameter in the cell membrane. *In vivo* such membrane lesions lead to swelling and lysis of the gut epithelium and death of the insect ensues through starvation and septicemia. Active δ -endotoxins of different specificities show five strongly conserved regions in their amino-acid sequences. Exchanging sequence segments in the divergent regions between toxins of different specificities can produce active hybrids showing altered target specificity. We have determined the atomic structure of a Coleoptera-specific δ -endotoxin (CryIII_A, beetle toxin) from B.t. subsp. *tenebrionis* to elucidate the structural basis for target specificity and membrane perforation by this family of proteins.

In summary, there is an extensive amount of information known in the art about the Cry endotoxins. While Applicants do not believe that such extensive characterization would be necessary in order to enable sequence identity claims, it seems clear that the amount of

⁶ Submitted herewith as Appendix A; submitted with the Amendment of 1/21/05 as Appendix A.

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information known in the art regarding Cry endotoxins should be deemed to provide sufficient guidance to one of skill in the art as to which regions of these proteins are more or less likely to tolerate mutations while still providing pesticidal activity.

On page 9 of the Office Action of 5/6/05 (first and second paragraphs), the Examiner concludes that it is not persuasive when Applicants argue that it is customary in the art to make and assay a number of sequences for a desired function in order to achieve the best results and that this type of experimentation is now considered routine in the art and would not be considered undue. Applicants disagree with this conclusion and are also confused by the Examiner's statements in these paragraphs.

Applicants thought they had made it clear that they were citing the Christians and Minshull references to illustrate generally that it is indeed customary in the art to make and assay a number of sequences in order to obtain sequences having a desired function. These references provide additional support regarding the state of the art. The Examiner seems to have misunderstood this and instead states that "it is unlikely that [a sequence from the Michaels reference] could be used to generate a nucleic acid that encodes a protein with 70% identity to SEQ ID NO:2." Applicants are confused as to why the Examiner is speculating as to the outcome of such experiments and are not sure how these statements are relevant to the present claims. Applicants are also confused by the last sentence of paragraph 2 on page 9 of the Office Action ("not predictable. could not be considered because they were not sent."). If there are additional points in these paragraphs to which Applicants should respond, clarification is requested.

Page 9 of the Office Action of 5/6/05 (third and fourth paragraphs) state that "undue trial and error experimentation would be required to make and assay vast numbers of nucleic acids in order to find any that fell within the scope of the claims," referring to the Lazar and Hill references. As discussed earlier, Applicants believe that this and similar statements illustrate that the Examiner is not applying the correct standard to the present claims. The enablement standard requires a disclosure to contain sufficient information to enable one skilled in the pertinent art to make and use the claimed invention (MPEP § 2164.01). Applicants reiterate that the amount of

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experimentation required to test a particular polypeptide for pesticidal activity would not be considered undue by one of skill in the art, as evidenced by the assay results presented in the specification, for example, in working Examples 4 (pp. 65-66), 6 (p. 67), and 7 (p. 69), and as evidenced by the Rule 132 declaration of Dr. André Abad⁷. Further, Applicants reiterate that the Lazar and Hill references mentioned in the Office Action (Lazar *et al.* (1988) *Mol. Cell. Biol.* 8: 1247-52 and Hill *et al.* (1998) *Biochem. Biophys. Res. Comm.* 244: 573-577) illustrate that one of skill would readily be able to determine whether a particular sequence change affected the function of a protein. Accordingly, one of skill in the art would be able to determine the functionality of polypeptides encompassed by the claimed invention without undue experimentation. In view of these statements and evidence, Applicants respectfully submit that the Examiner has not met her burden of establishing a reasonable basis to question the enablement provided for the claimed invention (MPEP § 2164.04) and therefore the rejection of the claims for lack of enablement should be reversed.

(b) Issue 2—Whether claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64 meet the written description requirement of 35 U.S.C. § 112, first paragraph.

In the final Office Action (5/6/05, page 10, #5), the Examiner rejected claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-65 under 35 U.S.C. § 112, first paragraph:

[A]s containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This rejection is repeated for the reasons of record.... Applicant's arguments...have been fully considered but they are not persuasive."

Claim 65 has been cancelled in an Amendment After Final filed herewith. Accordingly, this rejection will be discussed with regard to the remaining sequence identity claims. Because this rejection is repeated for the reasons of record, Applicants' arguments below are similar to those previously submitted.

The written description rejection in the final Office Action encompassed claims 1-3, 9-12, 17-19, 38, 43, 46, 49, 52, and 55-64. These claims contain limitations that require the

⁷ Submitted herewith as Appendix C; submitted with the Amendment of 1/21/05 as Appendix C.

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nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1 and thus are referred to herein as the "sequence identity claims." In maintaining this rejection, the Examiner disregards not only Applicants' arguments but also the case law cited in those arguments. Applicants respectfully submit that the Examiner is applying an extraordinarily high standard of written description to the present claims, a standard that is not properly based on case law or on the statute.

As an initial matter, Applicants note that the written description rejection in the final Office Action of 12/3/03 only referred to the claim limitation that requires nucleotide sequences to have at least 90% sequence identity to SEQ ID NO:1, but some of the claims included in that rejection specified at least 93%, 94%, and 95% sequence identity (*i.e.*, claims 55, 58, and 63 (93%), claims 56, 59, and 64 (94%), and claims 38, 43, and 49 (95%)). Applicants believe that some of these claims might be deemed to be allowable whereas others might not and therefore request that they be considered separately.

In this regard, Applicants also wish to clarify another issue raised by the Examiner. Applicants note that the Examiner refers throughout the Office Action to various numbers of nucleotide changes (*e.g.*, page 3, paragraph 4), almost as if the claims required that these numbers of nucleotide substitutions be made. Applicants wish to emphasize that the claims pending in the case contain limitations that require the nucleotide sequence of the claims to share a specified percent of sequence identity to SEQ ID NO:1, *i.e.*, at least 90% sequence identity. The claims do not, as the Examiner seems to suggest, require the modification of particular numbers of nucleotides (*see, e.g.*, page 11, second paragraph; paragraph bridging pages 11-12; paragraph bridging pages 12-13; page 13, second full paragraph).

Also, Applicants note that in the Office Action of 12/03/03 (page 4, #4, 3d paragraph), the Examiner stated that "nucleic acids that have 90% identity to SEQ ID NO:1 *are predictable*, nucleic acids that have 90% identity to SEQ ID NO:1 AND that encode pesticidal proteins are not" (emphasis added). Thus, the written description rejection was made on the grounds that there is inadequate description of sequences that both meet the sequence identity requirement of the claims and also meet the functional requirement (*i.e.*, that the encoded polypeptide has pesticidal activity).

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The claims meet the written description requirement as articulated by the Federal Circuit

Applicants respectfully submit that the present claims and specification meet the written description requirement of 35 U.S.C. §112, first paragraph, as clarified by *University of California v. Eli Lilly and Co.*, 119 F.3d 1559, 1569, 43 USPQ2d 1398 (Fed. Cir. 1997) and *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991); *cert. denied* 112 S.Ct. 169 (1991). Applicants have provided exemplary sequences of the invention as set forth in SEQ ID NO:1. Indeed, in the final Office Action of 5/6/05, the Examiner has indicated that claims drawn to the exemplary disclosed sequences (*i.e.*, claims 39, 40, 44, 45, 50, and 51) would be allowable if rewritten in independent form. The claimed nucleic acids are defined in relation to the exemplary disclosed nucleotide sequence of SEQ ID NO:1; that is, the claimed nucleic acids comprise nucleotide sequences that share a specified percentage of sequence identity with SEQ ID NO:1. Applicants have thus provided a structural definition of the sequences of the invention. Applicants have also provided assays by which those of skill in the art can readily assess whether a nucleic acid molecule meeting the nucleotide sequence element of the claims also meets the functional limitation element of the claims. This is what *Eli Lilly* requires, and Applicants have also conceived the sequences of the invention as articulated in *Amgen*; that is, Applicants are able "to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it." *Amgen*, 18 USPQ2d at 1021.

Applicants further note that the Federal Circuit has explicitly stated that:

Eli Lilly did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.

Amgen, Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1332, 65 USPQ2d 1385, 1398 (Fed. Cir. 2003). *See also, Mobu, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1320, 66 USPQ2d 1429, 1438 (noting that "[i]n more recent cases, however, this court has distinguished *Lilly*" and further noting that in *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.3d 956 (Fed. Cir.

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2002), "neither the specification nor the deposited biological material recited the precise 'structure, formula, chemical name, or physical properties' required by *Lilly*."

B. thuringiensis δ -endotoxins are well-known in the art, and further support is provided in the specification with working examples

As discussed extensively in the specification (e.g., on pp. 3, 7, 11-12, 15, 24-25), the disclosed exemplary sequence of SEQ ID NO:2 is a *Bacillus thuringiensis* Cry-8-like δ -endotoxin. The *B. thuringiensis* δ -endotoxins are an extremely well-characterized group of proteins. As discussed in the specification at pp. 24-25:

The inventors of the present invention used the **solved structure of the Cry3A gene** (Li et al. (1991) *Nature* 353: 815-821)⁸ to produce a homology model of the Cry8 δ -endotoxin disclosed and claimed herein as SEQ ID NO:2 to gain insight into the relationship between structure and function of the endotoxin, and to design the recombinantly engineered proteins disclosed and claimed herein. A combined consideration of the **published structural analyses of *B. thuringiensis* endotoxins** and the reported function associated with particular structures, motifs, and the like indicates that specific regions of the endotoxin are correlated with particular functions and discrete steps of the mode of action of the protein. For example, **δ -endotoxins isolated from *B. thuringiensis* are generally described as comprising three domains, a seven-helix bundle that is involved in pore formation, a three-sheet domain that has been implicated in receptor binding, and a beta-sandwich motif** (Li et al. (1991) *Nature*, 305: 815-821).

As discussed in more detail in the specification on p. 25, the inventors made use of this knowledge to design specific mutations in the Cry8-like proteins to enhance their pesticidal activity. This strategy was successful in creating altered endotoxins with increased toxicity, as demonstrated by the data presented in working example 6. Thus, the inventors were able, in view of the extensive knowledge in the art about *B. thuringiensis* δ -endotoxins, to modify the exemplary wildtype sequences disclosed herein to provide an endotoxin with enhanced pesticidal activity.

⁸ Submitted herewith as Appendix A; submitted with the Amendment of 1/21/05 as Appendix A.

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This knowledge in the art is also reflected in the Pfam alignments submitted by Applicants with the Amendment of 1/21/05⁹, which include detailed domain descriptions for each of "Endotoxin N," "Endotoxin M," and "Endotoxin C"; each of these descriptions cites the Li reference (Li *et al.* (1991) *Nature* 353: 815-821) discussed in the specification and discussed herein at length earlier as well as Schnepf *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62: 775-806, indicating that the critical information regarding these domains was known in the art well before the present application was filed.

In this manner, the data provided in the specification (e.g., Example 6) demonstrate that one of skill would know what amino acids could be changed to provide a protein with pesticidal activity. Accordingly, Applicants submit that adequate guidance is provided as to which amino acids of SEQ ID NO:2 are critical for function and that therefore, Applicants have envisioned the detailed construction of the gene to distinguish it from other materials, thereby meeting the written description requirement.

Applicants note that in the final Office Action of 12/3/03, the Examiner stated that "Applicant has not described even one nucleic acid that ... has 90% identity to SEQ ID NO:1 AND that encodes a pesticidal protein." Now in the final Office Action of 5/6/05 (page 12, last paragraph), the Examiner states that the fragments and variants taught in the specification "are not sufficient to describe nucleic acid [*sic*] within the full scope of the claims." Applicants respectfully disagree with these conclusions. The specification teaches a number of nucleic acids that share relatively low percent sequence identity with SEQ ID NO:1 but encode proteins having pesticidal activity. In Example 4 (specification pp. 65-66), both the full-length endotoxin encoded by SEQ ID NO:1 and a truncated protein encoded by SEQ ID NO:15 were assayed for pesticidal activity against southern corn rootworm. The nucleotide sequence of SEQ ID NO:15 is a truncation of SEQ ID NO:1 which shares about 55% sequence identity with SEQ ID NO:1. In Example 6 (specification pp. 67-69), several truncated proteins were assayed and shown to have pesticidal activity against Colorado potato beetle (see Table 1, p. 68). These truncated proteins included those encoded by SEQ ID NO:15 and SEQ ID NO:19, which share about 55%

⁹ Submitted herewith as Appendix B; submitted with the Amendment of 1/21/05 as Appendix B.

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and 51% identity, respectively, to the exemplary nucleic acid sequence set forth in SEQ ID NO:1 (alignments performed using BLAST with default parameters). Another mutant assayed for pesticidal activity in Example 6 was NGS1213-1 (encoded by SEQ ID NO:11). The NGS1213-1 mutant includes the amino acid sequence "NGSR" inserted between amino acids 164 and 165 of the truncated endotoxin of SEQ ID NO:16. The nucleotide sequence encoding this mutant (SEQ ID NO:11) shares about 56% sequence identity with the exemplary nucleotide sequence of SEQ ID NO:1, yet both encoded proteins have pesticidal activity. The specification also teaches an exemplary maize-optimized sequence (SEQ ID NO:9), which encodes the same pesticidal polypeptide as SEQ ID NO:15 but shares less than 69% sequence identity with it. Thus, the present specification provides multiple working examples illustrating the production of sequences that encode pesticidal proteins and share a relatively low percentage of sequence identity with SEQ ID NO:1. Multiple working examples are presented, illustrating that Applicants were in possession of the claimed invention at the time of filing.

In light of the above statements, Applicants respectfully assert that the present claims and specification satisfy the statutory written description requirement. Accordingly, Applicants respectfully request that the rejection of the sequence identity claims under 35 U.S.C. §112, first paragraph, be reversed.

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CONCLUSION

In view of the arguments presented above, Applicants contend that each of claims 1-3, 9-12, 17-19, 38-40, 43-46, 49-52, and 55-64 is patentable. Therefore, reversal of the rejections under 35 U.S.C. §112, first paragraph, is respectfully solicited.

If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefor (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

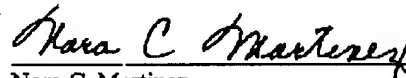


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Nora C. Martinez

9/6/05
Date

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CLAIMS APPENDIX

APPEALED CLAIMS

1. (Previously Presented) An isolated nucleic acid comprising a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1, wherein said nucleotide sequence encodes a polypeptide which is pesticidal for at least one pest belonging to the order Coleoptera.

2. (Original) The nucleic acid according to claim 1, wherein the nucleotide sequence is optimized for expression in a plant.

3. (Original) An expression cassette comprising a nucleic acid according to claim 1, wherein said nucleotide sequence is operably linked to a promoter that drives expression in a microorganism or in a plant cell.

4-8 (Canceled)

9. (Previously Presented) A transformed plant comprising in its genome at least one stably incorporated nucleotide construct comprising a nucleotide sequence encoding a polypeptide operably linked to a promoter that drives expression of said polypeptide, wherein said polypeptide is pesticidal for at least one pest belonging to the order Coleoptera and wherein said nucleotide sequence has at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1.

10. (Original) The plant according to claim 9, wherein the plant is a monocot.

11. (Original) The plant according to claim 9, wherein said plant is a dicot.

12. (Original) Transformed seed of the plant according to claim 9.

13-16 (Canceled)

17. (Previously Presented) A method for impacting an insect pest comprising introducing into a plant or cell thereof at least one nucleotide construct comprising a nucleotide

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sequence encoding a polypeptide operably linked to a promoter that drives expression of said polypeptide in plant cells, wherein said polypeptide is pesticidal for at least one pest belonging to the order Coleoptera and wherein said nucleotide sequence has at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1, whereby an insect pest feeding on said plant or cell thereof is impacted.

18. (Previously Presented) The method according to claim 17, wherein the plant produces a polypeptide characterized by pesticidal activity against at least one pest of the order Coleoptera.

19. (Previously Presented) The method according to claim 18, wherein said insect pest is selected from the group consisting of Colorado potato beetle, western corn rootworm, southern corn rootworm, and boll weevil.

20-37 (Canceled)

38. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence has at least 95% identity to the nucleotide sequence set forth in SEQ ID NO:1.

39. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence encodes the amino acid sequence set forth in SEQ ID NO:2.

40. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence is set forth in SEQ ID NO:1.

41-42. (Canceled)

43. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence has at least 95% identity to the nucleotide sequence set forth in SEQ ID NO:1.

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44. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence encodes the amino acid sequence set forth in SEQ ID NO:2.

45. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence is set forth in SEQ ID NO:1.

46. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence is optimized for expression in a plant.

47-48 (Canceled)

49. (Previously Presented) The method of claim 17, wherein said nucleotide sequence has at least 95% identity to the nucleotide sequence set forth in SEQ ID NO:1.

50. (Previously Presented) The method of claim 17, wherein said nucleotide sequence encodes the amino acid sequence set forth in SEQ ID NO:2.

51. (Previously Presented) The method of claim 17, wherein said nucleotide sequence is set forth in SEQ ID NO:1.

52. (Previously Presented) The method of claim 17, wherein said nucleotide sequence is optimized for expression in a plant.

53-54 (Canceled)

55. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence has at least 93% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1.

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56. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence has at least 94% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1.

57. (Previously Presented) The nucleic acid of claim 1, wherein said nucleotide sequence is optimized for expression in a plant.

58. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence has at least 93% sequence identity to the sequence set forth in SEQ ID NO:1.

59. (Previously Presented) The transformed plant of claim 9, wherein said nucleotide sequence has at least 94% sequence identity to the sequence set forth in SEQ ID NO:1.

60. (Previously Presented) The plant of claim 57, wherein said plant is a dicot.

61. (Previously Presented) The plant of claim 57, wherein said plant is a monocot.

62. (Previously Presented) The plant of claim 57, wherein said monocot is maize.

63. (Previously Presented) The method of claim 17, wherein said nucleotide sequence has at least 93% sequence identity to the sequence set forth in SEQ ID NO:1.

64. (Previously Presented) The method of claim 17, wherein said nucleotide sequence has at least 94% sequence identity to the sequence set forth in SEQ ID NO:1.

65. (Canceled)

EVIDENCE APPENDIX

APPENDIX A

ARTICLES

42. Porter, N. A. & Weekes, T. C. *Mol. Biol. Evol.* 183, 205-210 (1978).
 43. Akai, C. W. et al. in *Proc. 21st ICRG, Adelaide*, vol. 2 (ed. Prothero, R. J.) 135-138 (Adelaide University, 1980).
 44. Porter, N. A. & Weekes, T. C. *Asmophy. J.* 213, 224-226 (1977).
 45. Porter, N. A. & Weekes, T. C. *Mol. Biol. Evol.* 183, 205-210 (1978).
 46. Porter, N. A. & Weekes, T. C. *Nature* 277, 193 (1979).
 47. Nolan, K., Porter, N. A., Fegan, D. J., Chantoli, M. Z. & Weekes, T. C. *Proc. 21st ICRG Adelaide*, vol. 2 (ed. Prothero, R. J.) 150 (Adelaide University, 1980).
 48. Fegan, D. J., McInerney, B., O'Brien, O. & O'Sullivan, C. *Nature* 273, 731-732 (1978).
 49. Blum, P. N. et al. *Nature* 204, 433-434 (1964).
 50. Porter, N. A. & Weekes, T. C. *Nature* 267, 507-508 (1977).

51. O'Meara, E. *Nature* 242, 130-137 (1973).
 52. Higuera, G. R. & Moore, G. L. *Asmophy. J.* 187, 157-158 (1974).
 53. Pinner, S. & Taylor, J. H. *Nature* 277, 117-120 (1979).
 54. Reed, M. J. *Nature* 256, 331-334 (1977).
 55. Kelly, A. V., Juhász, G. A. & O'Meara, E. *Nature* 267, 400-400 (1977).

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Crystal structure of insecticidal δ -endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution

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The structure of the δ -endotoxin from *Bacillus thuringiensis* subsp. *tenebrionis* that is specifically toxic to Coleoptera insects (beetle toxin) has been determined at 2.5 Å resolution. It comprises three domains which are, from the N- to C-termini, a seven-helix bundle, a three-sheet domain, and a β sandwich. The core of the molecule encompassing all the domain interfaces is built from conserved sequence segments of the active δ -endotoxins. Therefore the structure represents the general fold of this family of insecticidal proteins. The bundle of long, hydrophobic and amphipathic helices is equipped for pore formation in the insect membrane, and regions of the three-sheet domain are probably responsible for receptor binding.

THE δ -endotoxins are a family of insecticidal proteins produced by *Bacillus thuringiensis* (B.t.) during sporulation, having relative molecular masses (M_r) 60,000–70,000 (60K–70K) in the active form and specific toxicities against insects in the orders of Lepidoptera, Diptera and Coleoptera^{1,2}. These toxins have been formulated into commercial insecticides for three decades³, and now insect-resistant plants are engineered by transformation with Lepidoptera-specific toxin genes^{4–6}. In the bacterium δ -endotoxins are synthesized as protoxins of M_r 70K–135K and crystallize as a parasporal inclusion $\sim 1 \mu$ in size, in which form they are ingested by the susceptible insect. The microcrystal dissolves in the alkaline pH of the midgut and the protoxin is cleaved by gut proteases to release the active toxin. δ -Endotoxins activated *in vitro* bind specifically and with high affinity ($K_D = 0.1$ –20 nM) to protein receptors on brush-border membrane vesicles derived from the gut epithelium of target insects^{7–9} and create leakage channels of 10–20 Å diameter in the cell membrane¹⁰. *In vivo* such membrane lesions lead to swelling and lysis of the gut epithelium¹¹ and death of the insect ensues through starvation and septicaemia. Active δ -endotoxins of different specificities show five strongly conserved regions in their amino-acid sequences^{1,12}. Exchanging sequence segments in the divergent regions between toxins of different specificities can produce active hybrids showing altered target specificity^{13–15}. We have determined the atomic structure of a

Coleoptera-specific δ -endotoxin (CryIIIA, beetle toxin) from B.t. subsp. *tenebrionis*^{16–18} to elucidate the structural basis for target specificity and membrane perforation by this family of proteins.

Structure determination

Parasporal crystals of the beetle toxin contain the full-length 644-residue protoxin¹⁷ as the minor component, and a product of bacterial processing with 57 residues removed from the N-terminus as the major component¹⁹. The latter (M_r 67K) is similar in sequence to the active form of other δ -endotoxins. After solubilization, papain cleavage converts the mixture to the 67K toxin (see legend to Table 1). This was recrystallized in the original crystal form of the parasporal crystals, space group C222, and cell dimensions 117.1 by 134.2 by 104.5 Å, containing one molecule per asymmetric unit and 55% solvent by volume¹⁸.

Initial evaluation of derivatives was carried out at 4.5 Å resolution with data collected on the FAST TV diffractometer²⁰ using CuK α radiation. Complete datasets (Table 1) were then collected to 2.5 Å resolution from native crystals using the imaging plate systems at the EMBL outstation at DESY and from the mercury and platinum derivatives on film at SRS Daresbury. The electron density map (Fig. 1) at 2.5 Å resolution calculated with phases from multiple isomorphous replacement (mean figure of merit, 0.63) was easily interpretable and was improved by solvent flattening^{21,22}. A continuous polypeptide chain from residue 61 to residue 644 at the C terminus was traced unambiguously, and most side-chain atoms could be located in the map. The atomic model was built using the graphics program χ (ref. 23) and had an initial *R*-factor of 37% for all data to 2.5 Å. After preliminary refinement using the program X-PLOR (ref. 24), the current model, containing 584 amino acid residues and 40 bound water molecules, has an *R*-factor of 19.9% and i.m.s. bond length deviation of 0.017 Å.

Description of the structure

Overview. The beetle toxin is a wedge-shaped molecule with a radius of gyration of 58 Å. As shown in Fig. 2a, it comprises three domains. Domain I, from the N terminus of the 67K toxin to residue 290, is a seven-helix bundle in which a central helix is completely surrounded by six outer helices tilted at about $\pm 20^\circ$ to it (Fig. 3b,c). Domain II, from residues 291 to 500, contains three antiparallel β sheets packed around a hydrophobic core with a triangular cross-section (Fig. 4). Domain III, from residues 501 to 644 at the C terminus is a sandwich of two antiparallel β sheets (Fig. 5). Domains I and II make up the

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TABLE 1 Data collection and phasing statistics

Data collection						
Data	Method of collection	Number of crystals	Resolution (Å)	Number of measurements	Unique reflections (% completeness)	R_{merge}
Native	image plate	8	2.5	121,767	27,727 (100)	0.108
CH_3HgNO_3	film	7	2.5	103,623	27,767 (100)	0.095
$\text{Hg}(\text{CH}_3\text{COO})_2$	film	5	2.5	60,224	25,919 (94.5)	0.103
<i>cis</i> - $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$	film	7	2.5	86,629	25,924 (94.5)	0.107
K_2OsO_4	FAST	1	4.5	21,143	4,680 (100)	0.077
HgCl_2	FAST	1	4.5	20,013	4,701 (100)	0.069
Phasing statistics						
Derivative	Anomalous data	Number of sites	$R_{\text{deriv}}^{\dagger}$	$R_{\text{calc}}^{\ddagger}$	Phasing power [§] (resolution, Å)	
CH_3HgNO_3	no	3	0.183	0.715	1.5(1) (2.5)	
$\text{Hg}(\text{CH}_3\text{COO})_2$	yes	6	0.247	0.609	2.2(1) (2.5)	
<i>cis</i> - $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$	no	5	0.185	0.682	1.5(1) (2.5)	
K_2OsO_4	no	4	0.149	0.757	1.2(1) (5.5)	
HgCl_2	no	3	0.095	0.741	1.3(1) (5.0)	

Protein preparation: Solubilized porcupine crystals from *B. l. subsp. tenebrionis* were incubated at 0.5 mg ml⁻¹ protein with 0.125 units per ml Agarose-linked papain (Boehringer) in 3.3 M NaBr, 0.05 M sodium phosphate, pH 7.0, and 0.1 mg ml⁻¹ phenylmethylsulphonyl fluoride (PMSF) for 30 min at 20 °C. Digestion was stopped by adding vinyl lysinechloromethylketone (TLCK) to 0.125 mg ml⁻¹ and Na₂CO₃ to one fifth volume and removing it by dialysis. The 67 kDa toxin was then purified by gel filtration on Sephadex G75 equilibrated with 0.1 M NaHCO₃, pH 10.5, 0.5 M NaBr. Crystallization: Single crystals were obtained by microdialysis at a protein concentration of 2.5 mg ml⁻¹ against 0.1 M NaHCO₃, pH 9.2, 0.5 M NaBr at 16 °C; 3 mM NaN₃, 0.1 mM PMSF and 0.1 mg ml⁻¹ TLCK were present in all buffers. Crystals were transferred by stages to 0.05 M 2-(N-morpholino)ethanesulphonic acid (MES), pH 6.5, for derivative preparation and mounted in 0.03% low-melting agarose in this buffer during data collection. Data collection: Image plate and film data were processed using MOSFLM (Imperial College, London) and CCP4 programs (Daresbury Lab). FAST (ref. 20) data were collected and processed with MADNES¹⁶, and scaled in 3° batches. Derivatives: Crystals were soaked respectively in 0.25 M CH_3HgNO_3 for 3.6 h, in 1 mM $\text{Hg}(\text{CH}_3\text{COO})_2$ for 14 h, in freshly prepared 1 mM *cis*- $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ for 21 h, in saturated K_2OsO_4 for 35 h, and in 2 mM HgCl_2 for 3 days. Phase calculation: Two heavy-atom sites in each derivative were located from difference Patterson functions, except in the case of $\text{Hg}(\text{CH}_3\text{COO})_2$ for which 3 sites were located, and the remaining sites were found by cross-phased difference Fourier. Heavy-atom parameters were refined against centric data and phases calculated for all data using the program PHARE (G. Bricogne). The two low-resolution derivatives were refined against phase calculated from the high-resolution derivatives. Phasing with the three high-resolution derivatives gave an overall figure of merit of 0.61 (2.5–2.5 Å) and clearly interpretable map. Including the remaining derivatives slightly improved the connectivity of the map (overall figure of merit 0.63), and four cycles of solvent flattening using a 50% solvent content and a 9 Å radius in mask calculation^{21,22} improved the overall definition of densities. The starting model was built using the program O (ref. 23) with the Bones option for main-chain tracing and the autobuild and manip options for side chains. Refinement: simulated annealing using the program X-PLOR (ref. 24) reduced the R -factor from 0.37 to 0.25 without individual B -factors, and to 0.23 with restrained individual B -factors. The model was adjusted in the loops 154–156, 429–436, and 483–488, and had 40 solvent molecules added, then refined by X-PLOR again. The current model has an R -factor of 19.9%, with r.m.s. bond length deviation of 0.017 Å, r.m.s. bond angle deviation of 3.2°, and average atom B -factor of 18 Å².

[†] $R_{\text{merge}} = \sum_i \sum_j |I_i - \langle I \rangle| / \sum_i \langle I_i \rangle$, where I_i are intensity measurements for a reflection, and $\langle I \rangle$ is the mean intensity for this reflection.

[‡] $R_{\text{calc}} = \sum_i |F_{\text{calc}} - F_{\text{obs}}| / \sum_i |F_{\text{calc}}|$, where F_{calc} is the structure factor amplitude of the derivative crystal and F_{obs} is that of the native.

[§] $R_{\text{calc}} = \sum_i (|F_{\text{calc}}| - |F_{\text{obs}}|) / \sum_i |F_{\text{calc}}|$, where F_{calc} and F_{obs} are defined as for R_{deriv} , and F_{calc} is the calculated heavy-atom structure factor amplitude summed over centric data only.

[§] Phasing power = $(F_{\text{calc}})/E$, the r.m.s. heavy-atom structure factor amplitudes divided by the residual lack of closure error.

bulky end of the molecule. Through their contact one of the two β sheets in domain III is almost entirely buried. To our knowledge (see, for example, ref. 25), the packing of helices in domain I and of sheets in domain II are both novel arrangements.

Domain I. The central helix in this seven-helix bundle is α_3 (Fig. 3b,c), which is oriented with its C terminus towards the bulky end of the molecule. Viewed from this end, the outer helices are arranged anticlockwise in the order of α_1 , α_2 , α_4 , α_6 and α_7 , with helices α_1 and α_7 adjacent to the β -sheet domains; α_3 is interrupted by a non-helical section and only the leading half, α_{3a} , is packed against α_7 . Figure 3a shows the alignment of amino-acid sequence on the surfaces of the helices. The helices are long, especially α_3 to α_7 , which contain respectively 8, 7, 6, 9 and 7 complete helical turns and hence would be long enough to span the 30-Å thick hydrophobic region of a membrane bilayer. Furthermore, the six outer helices bear a strip of hydrophobic residues (defined by $\Delta G \geq 0$ for transfer from oil to water) down their entire length on the side-facing helix α_3 , so they are amphipathic. In keeping with the general observation that secondary structures are close-packed and bury hydrophobic surfaces²⁶, the helix contact angles in this domain cluster around +20° rather than -50°, giving the bundle a bouquet-like appearance (Fig. 3b). Figure 3c shows the bundle in cross-section. The interhelical space contains 27 aromatic residues which are packed in the edge-to-face fashion²⁷; all polar groups in this region are hydrogen-bonded or in salt bridges.

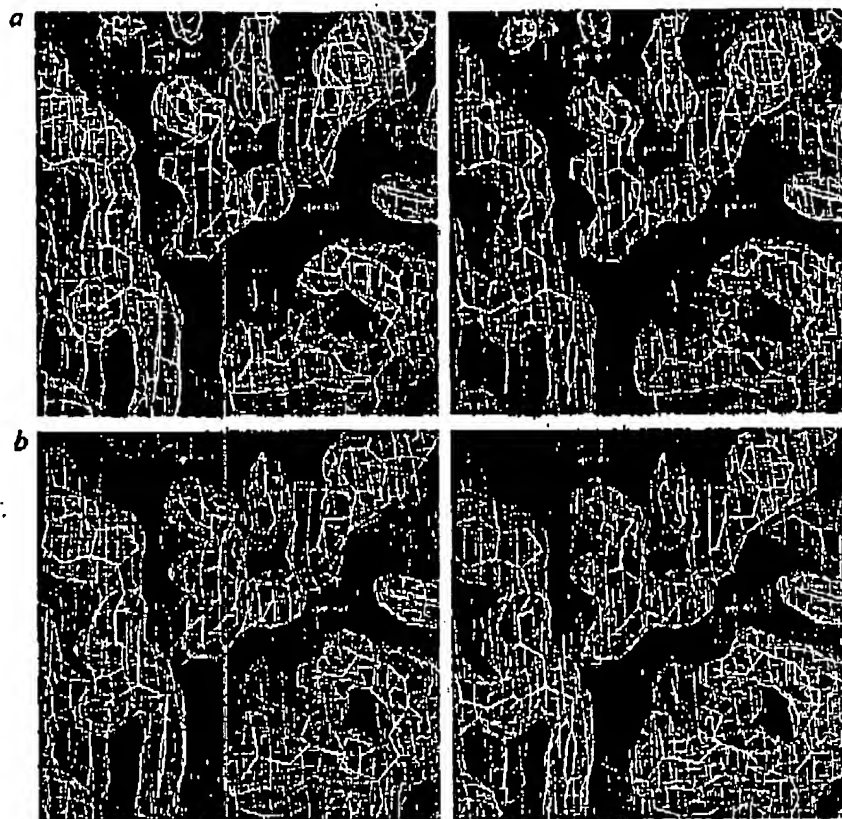
The concentric arrangement of the seven-helix bundle is distinct from the two-layered type seen in bacteriorhodopsin. There is some resemblance to the pore-forming domain of colicin A²⁸ in which two hydrophobic helices are shielded from solvent by eight amphiphilic helices, but the colicin helices are generally shorter. Like the colicin helices, the bundle in the bacteric toxin may be a soluble form of packaging for the hydrophobic and amphiphilic helices that will form pores in the membrane after a large change in conformation.

Domain II. In Fig. 4a and 4b the three sheets of this domain are laid side-by-side, as they would be seen from the solvent. There is an apparent structural duplication between the four-stranded antiparallel sheets, sheet 1 and sheet 2. The chain connections β_4 , β_3 , β_2 , β_5 and β_6 , β_7 , β_8 , β_9 , respectively, follow the order of +3, -1, -1, +3, which is typical of the 'Greek-key' topology²⁹. From both sheets the inner strands, β_1 and β_2 as well as β_7 and β_8 , extend some 20 Å to the apex of the molecule as two-stranded β ribbons; and at the point of departure from the sheets there is a β -bulge in β_3 and in β_7 to twist the plane of the ribbon by nearly 90° relative to the sheet. The connections between the outer strands cross over the ribbons on the solvent side.

The pseudo-symmetry between these sheets is very approximate. Using the least squares option in O (ref. 23), the sheet region of the strands β_3 and β_7 can be brought to superimpose on that of β_1 and β_8 , with a r.m.s. fit of 0.72 Å for 13 α carbons. But the r.m.s. fit increased to 1.1 Å for 22 α carbons of the

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FIG. 1 Electron density map in the neighbourhood of Cys 243, calculated *a*, using combined phases⁴⁰ from multiple isomorphous replacement and solvent flattening, and *b*, using combined experimental and model phases⁴⁸ after refinement by X-PLOR. The refined structure is shown superimposed for reference. Although Cys 243 is a major site of both the methylmercury (MM) and mercuric acetate (MA) derivatives, the methyl mercury site is in a hydrophobic environment compared with the mercuric acetate site.



whole inner strands including the ribbon region, and 1.7 Å for 36 α carbons on all four strands. Nonetheless, the sequence alignment brought by this superposition of the two sheets revealed a low level of internal homology, with seven pairs of equivalent residues (shown in bold) out of 41 aligned α carbons:

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338 IDIQFPIIRFQP(6)GFYWS(1)NYVETBPSI(0)OSHD117SPY(10)RLKWP 395
402 AVANTHLATWP(0)SAVTSO(1)TKVZRFQYV(3)DEASTQTVDS(7)SQDRI 453
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The three-stranded sheet 3 is formed by two separate polypeptide segments. The C-terminal segment of domain I contributes the two-stranded ribbon of β_{10} and β_{11} , whereas the N-terminal segment of this domain contributes strand β_1 , which is hydrogen-bonded to β_{11} ; β_1 is followed by a two-turn helix α_8 and an extended chain.

Figure 4c and d shows in side view and in cross-section that the three antiparallel sheets are packed around a triangular hydrophobic core. This brings the strand β_{10} on the edge of sheet 3 into proximity with strand β_4 on the edge of sheet 1, as well as placing the loops at the end of the three β ribbons into a region of about 12 Å radius at the molecular apex. This domain is in contact with helix α_7 of domain I on the face of sheet 3 (Fig. 4c).

Domain III. Figure 5 is a ribbon drawing of the strands forming the two sheets of the β sandwich. The sheet containing the C-terminal strand is in contact with domain I and will be called the inner sheet. This domain has the 'jelly-roll' topology²⁰, because it can be generated by folding an antiparallel β ribbon which starts with β_{13} (N terminus) and β_{23} (C terminus) on the inner sheet, and ends in the loop between β_{16} and β_{10} on the outer sheet; β_{14} is a short excursion from this ribbon and forms the fifth antiparallel strand of the outer sheet. In addition, small parallel sheets are formed at the edge of the β sandwich through hydrogen bonding of strand β_{12} to β_{16} at the edge of the outer sheet, and β_1 to β_{11} at the edge of the inner sheet.

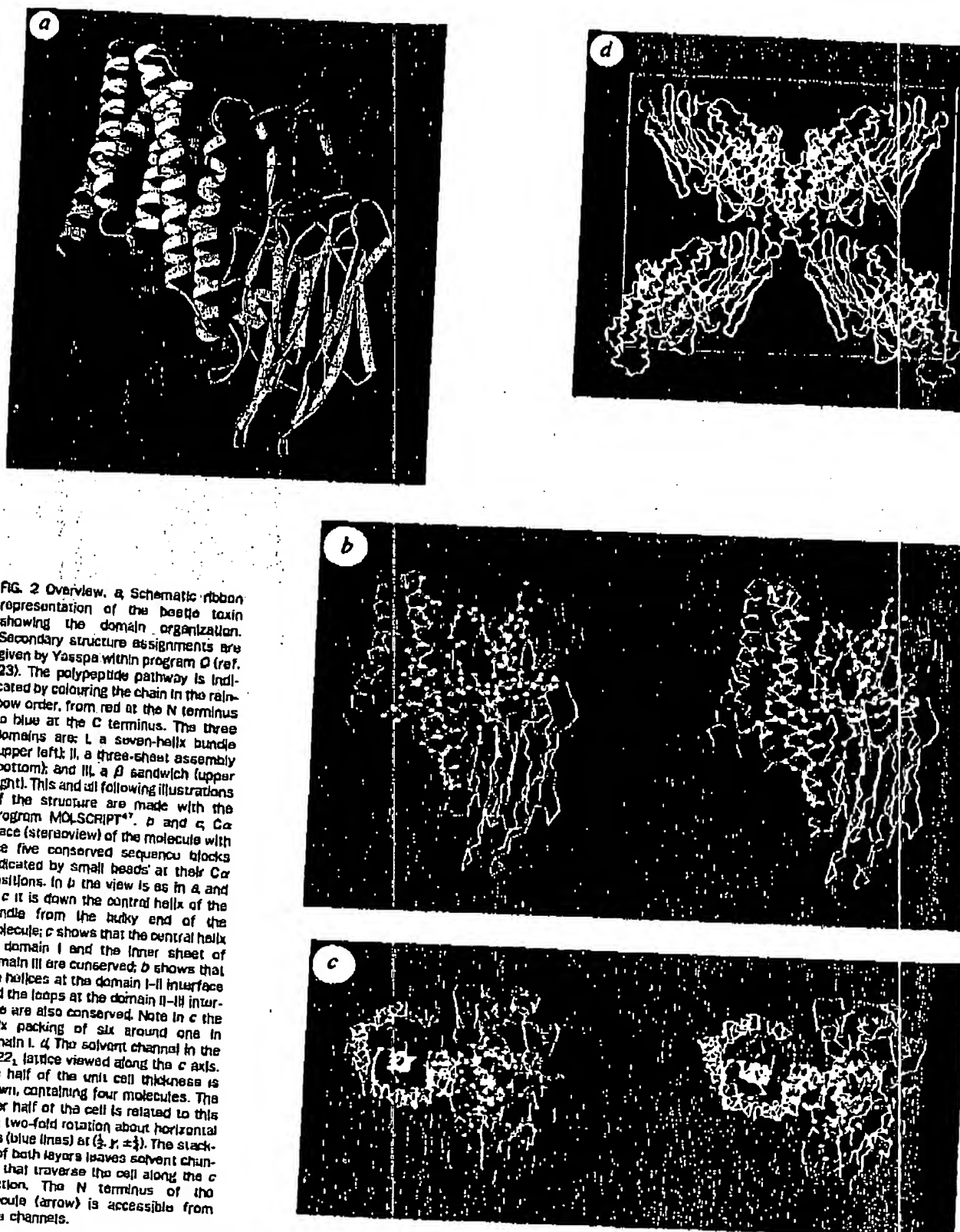
Distribution of conserved sequences. The core of the beetle toxin molecule encompassing the domain interfaces is built from the five sequence blocks that are highly conserved throughout the δ -endotoxin family¹ (Fig. 2b,c). Block 1, located in the beetle toxin sequence at residues 189–218, corresponds to the central helix (α_5) of the bundle in domain I. Block 2, residues 239–305, overlaps with the latter half of α_6 , and with α_7 and β_1 ; the latter hydrogen-bonds to the edge of the inner sheet in domain III before forming part of the three-stranded sheet 3 in domain II. Block 3, residues 491–538, overlaps with the latter part of β_{11} , where it is hydrogen-bonded to β_1 , and with the loops connecting domains II and III. The remainder of block 3 together with blocks 4 and 5, namely residues 560–569 and 633 to the C terminus, respectively, constitute the three buried strands of the inner antiparallel sheet in domain III. The high degree of conservation of internal residues implies that homologous proteins would adopt a similar fold. Using the beetle toxin structure as a model, we can therefore propose a basis for the insecticidal activity of δ -endotoxins as a family.

Basis of insecticidal function

Solubility. The beetle toxin crystals are isomorphous with the parasporal crystals^{18,19} and show the molecular contacts responsible for solubility behaviour *in vivo*. Four intermolecular salt bridges, Asp 142–Arg 165, Asp 224–Arg 562, Asp 590–Arg 178, and Glu 223–Lys 293, are located at contacts to three different neighbouring molecules. Such salt bridges keep the protoxin crystals insoluble until exposed to the extreme pHs in the insect midgut.

Proteolytic activation. Pro- δ -endotoxins have M_s of either ~130K or ~70K. Activation by larval gut proteases removes the C-terminal half of the larger protoxins^{20,21} and cleaves them at residue 28 or 29 from the N terminus. The smaller protoxins, such as that of the beetle toxin, are processed only at the N

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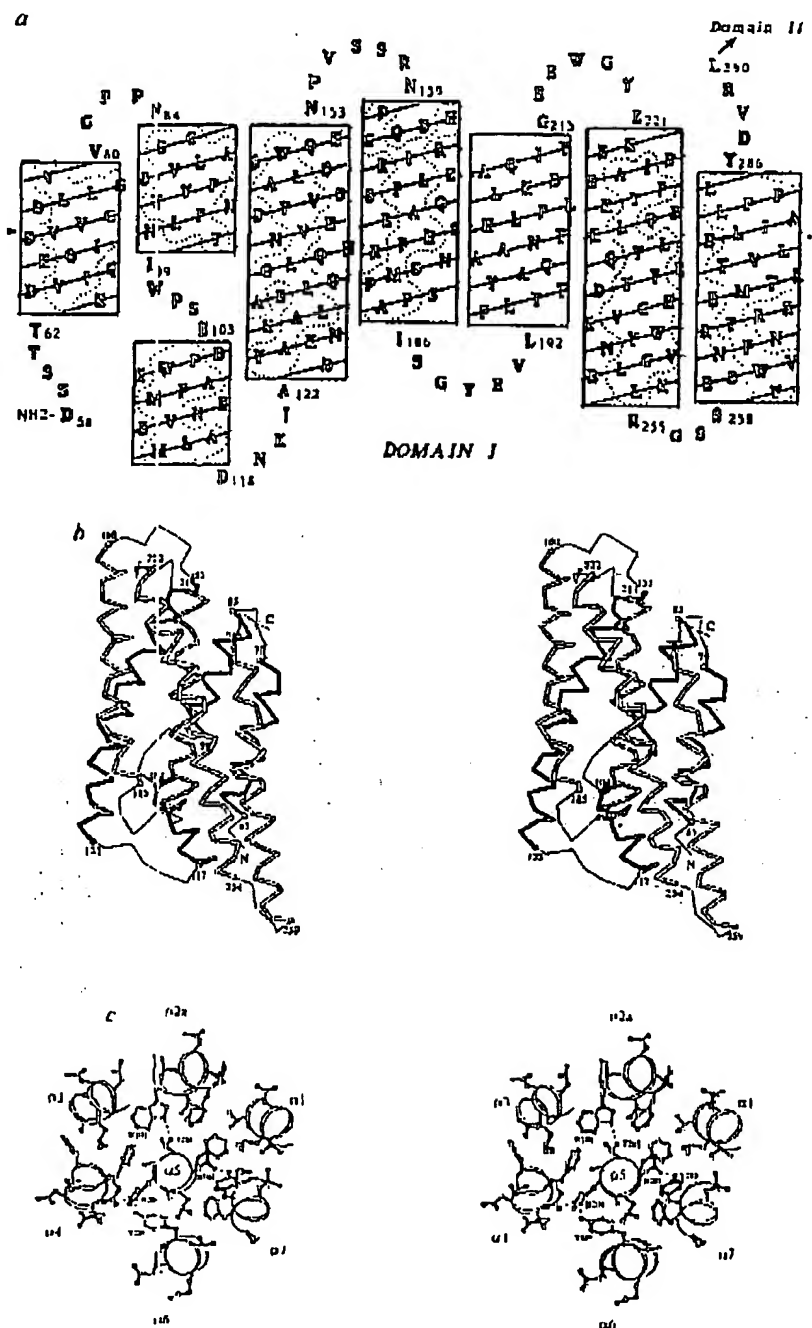


FIG. 3 The seven-helix bundle. **a** Helical nets showing the position of amino-acid residues along the 7 helices: α_1 (63-70), α_2 (α_{2a} , 65-88 and α_{2b} , 104-117), α_3 (123-152), α_4 (160-185), α_5 (193-214), α_6 (222-254) and α_7 (259-285). The cylindrical surface of the helices are cut longitudinally on the side facing the solvent and flattened to give a view from the interior of the bundle. The top of the drawing corresponds to the bulky end of the whole molecule. Owing to tilting of the outer helices, different helices are in register vertically only at a level indicated by two arrows pointed at α_4 and α_7 ; α_6 is the central helix. Dotted curves outline the strip of hydrophobic residues down the inward surface of the other six helices. **b** C_α trace (stereoview) for the bundle viewed perpendicular to α_6 . The relative tilt of the outer helices to α_6 and that between adjacent outer helices are both about 20° . The C_α trace is shaded grey over helices α_1 to α_3 in the back, striped over helix α_5 in the centre, and white over helices α_4 , α_6 , and α_7 in the front. **c** Cross-section of the bundle at the level indicated by the arrows in **a** viewed from the bulky end of the molecule. The helical backbone is represented by curly ribbons passing through the C_α positions. The outer helices are positioned roughly hexagonally around the central one and tilted relative to it, so the bundle forms a left-handed superhelix. The aromatic side chains are packed in an edge-to-face fashion. Hydrogen bonds are shown for side-chain atoms.

terminus^{14,22} where about 50 residues are removed. The activated δ -endotoxins show a conserved C-terminus, so-called sequence block 5 (ref. 1). Its position as the middle strand of the buried β sheet in domain III precludes further processing from the C terminus. In fact deletion from this site by 4 to 8 residues results in inactive mutants with altered solubility and immunogenicity^{20,22-25}. This is not surprising as the inner sheet can be expected to play a critical part in the structural integrity and stability of the toxins through interaction with the helical bundle.

At the N-terminal cleavage sites the different protoxin sequences show locally similar hydrophobic profiles^{16,37}, which would be consistent with a common topology for the N-terminal region of the activated toxins as seen in the helical bundle of

the beetle toxin. In crystals of the beetle toxin, the N terminus at the start of helix α_1 borders on a large solvent channel of about 30 Å diameter that crosses the unit cell along the c direction (Fig. 2d). This channel could allow access of sporulation-associated proteases to the cleavage site in parasporal crystals¹⁴. **Receptor binding.** The insecticidal selectivity of δ -endotoxins is due to high-affinity binding to specific membrane receptors^{7-9,38}, which in three cases seem to be glycoproteins¹⁸⁻²⁰. For several δ -endotoxins the specificity-determining regions have been delimited by exchanging sequence segments between closely related toxins of differing specificities¹³⁻¹⁵. Guided by the location of secondary structures in the beetle toxin, a plausible alignment of δ -endotoxin sequences was made for the non-

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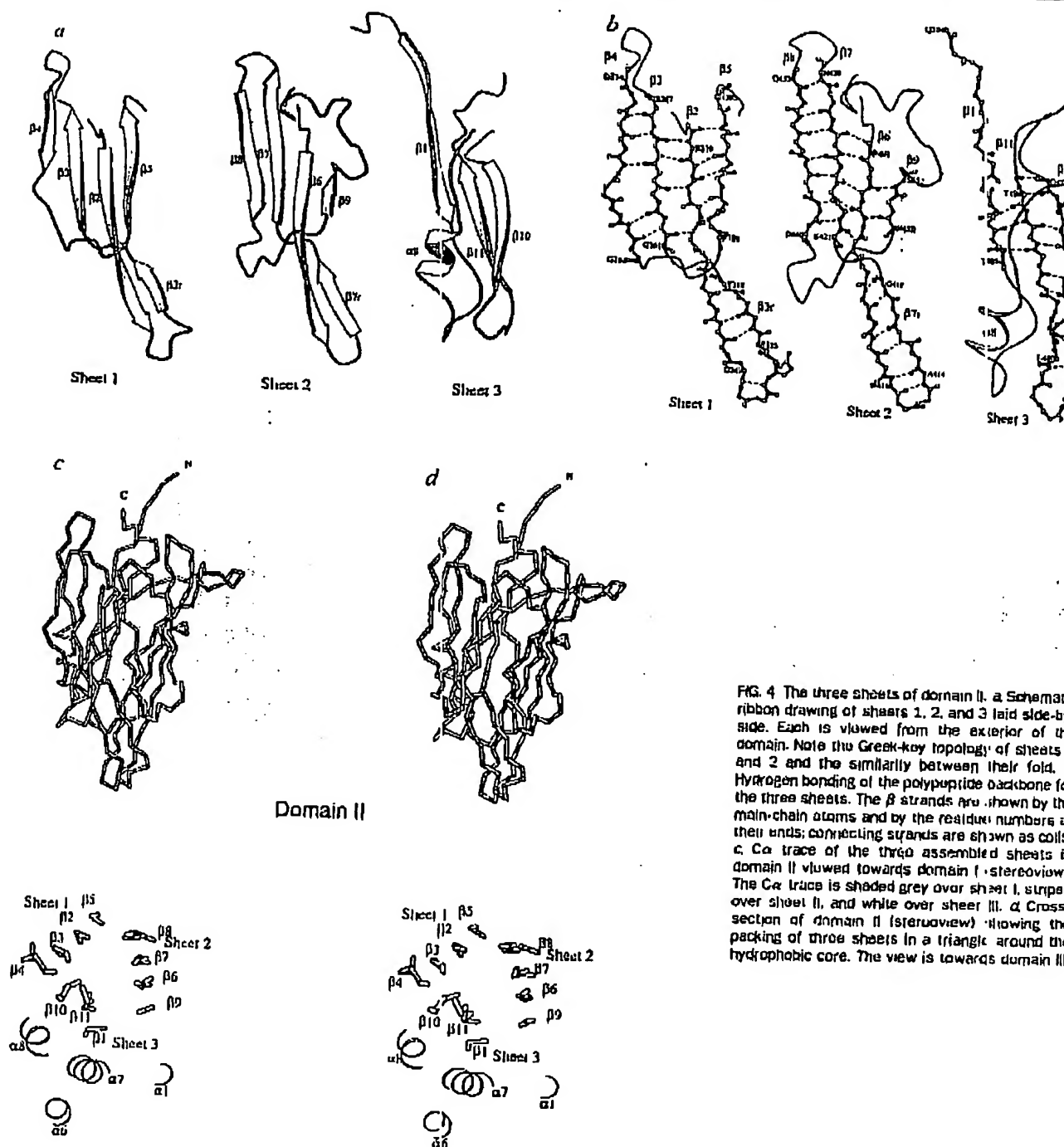


FIG. 4 The three sheets of domain II. a Schematic ribbon drawing of sheets 1, 2, and 3 laid side-by-side. Each is viewed from the exterior of the domain. Note the Greek-key topology of sheets 1 and 2 and the similarity between their fold. b Hydrogen bonding of the polypeptide backbone for the three sheets. The β strands are shown by the main-chain atoms and by the residue numbers at their ends; connecting strands are shown as coils. c Ca trace of the three assembled sheets in domain II viewed towards domain I (stereoview). The Ca trace is shaded grey over sheet I, striped over sheet II, and white over sheet III. d Cross-section of domain II (stereoview) showing the packing of three sheets in a triangle around the hydrophobic core. The view is towards domain III.

conserved regions (ref. 12, and T. C. Hodgman, unpublished results). Hence the genetically identified specificity-determining regions can be mapped to equivalent positions in the beetle toxin structure, and these fall mainly in domain II. For instance, the dual specificity of CryIIA for Lepidoptera and Diptera, as distinct from the Lepidoptera specificity in the closely related CryIIB, is determined by residues 307-382 of their sequences¹⁴, which corresponds roughly to sheet 1 (Fig. 4a) plus strand β_6 in sheet 2 and the loop leading up to β_7 , whereas the Lepidoptera

specificity of CryIIB is dependent on a longer segment¹⁴ that would include both inner strands of sheet 2. Similarly, the toxicities of CryIA(n) and CryIA(c) to two lepidopteran insects depend on three segments termed x, y and z (ref. 15): amino-acid substitutions in y can reduce toxicity by up to 2,000-fold, and segments x and y interact in determining specificity. Aligned with the beetle toxin structure, segment x corresponds roughly to the outer strands β_4 and β_5 of sheet 1 and the whole of sheet 2, including the loop entering β_{10} in sheet 3; y corresponds to

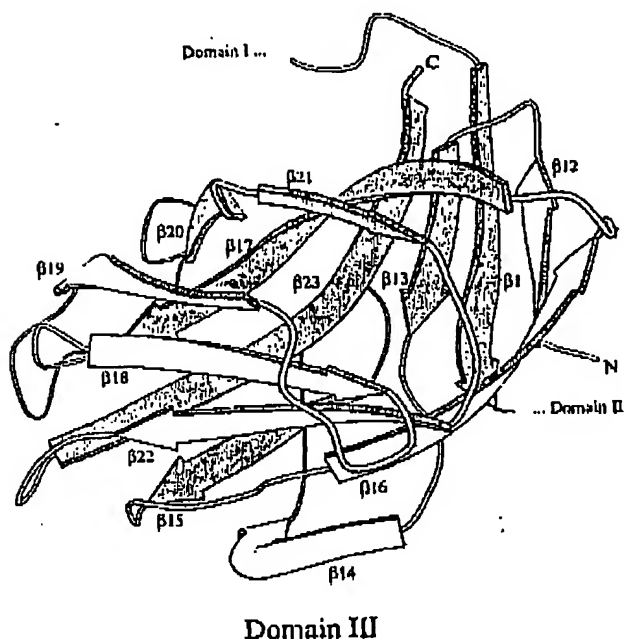


FIG. 5 Domain III, schematic ribbon representation of the β sandwich. β strands forming the inner sheet are shaded gray. The topology of an eight-stranded 'jelly-roll' can be seen by following the β hairpin starting with β_{12} , β_{11} and β_{10} in the inner sheet, continuing to β_9 and β_8 in the outer sheet, then β_7 and β_6 , β_5 in the inner sheet, and ending with β_4 and β_3 in the outer sheet. β_4 is an excursion from the hairpin and forms a fifth antiparallel strand of the outer sheet. Small parallel β sheets are added to one edge of the β sandwich, by hydrogen bonding of β_1 to β_{12} in the inner sheet and β_{11} to β_6 in the outer sheet. Residue numbers in the β strands are: β_{12} , 602-506; β_{11} , 509-513; β_{10} , 519-525; β_9 , 536-541; β_8 , 547-554; β_7 , 558-566; β_6 , 573-579; β_5 , 585-591; β_4 , 604-609; β_{11} , 611-614; β_{10} , 619-625; and β_{12} , 631-643.

strand β_{10} of sheet 3 and the loop connecting β_{10} and β_{11} ; and α extends from β_{11} to the C-terminal activation site. Furthermore, the interaction between α and γ can be understood in terms of the proximity between β_4 on the edge of sheet 1 and β_{10} on the

edge of sheet 3. Although α was inferred¹⁵ to extend into domain III, the combined evidence from genetics and receptor-binding assays *in vitro* for Lepidoptera toxins^{9,41} correlates receptor recognition with sequence variations within domain II. We note that the β ribbons from all three sheets terminate in loops in a small region on the molecular apex, in a manner reminiscent of the complementarity-determining region of immunoglobulins.

Pore formation. The common mechanism of epithelial cell disruption by δ -endotoxins of widely different specificities is believed to be the formation of lytic pores of 10 to 20 Å diameter in the insect membrane¹⁰. The structure of the beetle toxin displays an apparatus for pore formation in the long, hydrophobic and amphipathic helices of domain I which could penetrate the membrane. Between the crystal structure in which the bouquet-like helical bundle internalizes all the hydrophobic surfaces, and the unknown pore structure where hydrophobic surfaces would be in intimate contact with the membrane lipids, large conformational changes must occur. In the absence of a full characterization of the pore-forming process, we propose the following by extrapolation from the crystal structure.

The trigger for the conformational changes may be provided by receptor binding and the consequent interaction of toxin with the membrane bilayer. Membrane insertion follows rapidly, so that a major part of the bound δ -endotoxin cannot be displaced from the brush-border vesicles by other toxins recognizing the same receptor sites^{7,9}. As domain II and probably its apical region are most likely to bind the membrane receptors, the helices are expected to insert with the 'domain II end' (see Fig. 2a) oriented towards the cytoplasm. If helical hairpins are to initiate the membrane penetration, as probably happens for colicin^{28,42,43}, they will probably be linked at the domain II end. So either of the helix pairs α_2 - α_3 or α_1 - α_5 could be the likely initiator. The α_2 - α_3 pair is favoured because it forms part of the conserved interface with domain II and is well positioned to sense the receptor binding. On the other hand, helix α_5 is the most conserved throughout the family of δ -endotoxins. Point mutations in α_5 reduce toxicity of a Lepidoptera toxin without reducing binding to membranes⁴⁴. Proteolysis in the interhelical loops at the domain III end, as in the α_1 - α_4 loop^{19,32}, may facilitate release of the helix pairs from the tertiary structure of the bundle. The insertion of a hairpin can create a defect in the membrane, allowing the rest of domain I to participate in pore formation in a cooperative manner. □

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1. Höfte, H. & Whitlady, M. N. *Microbiol. Rev.* 53, 242-255 (1989).
2. Eiler, D. J. et al. in *Molecular Biology of Membrane Differentiation* (eds Hirth, L. A. & Setlow, P.) 230-240 (Am. Soc. Microbiol., Washington, DC, 1989).
3. Wilson, C. R. et al. in *Protein Engineering: Applications in Science, Medicine and Industry* (eds Inoué, M. & Saito, N.) 395-413 (Academic, New York, 1989).
4. Vaché, M. et al. *Nature* 328, 33-37 (1987).
5. Perletti, R. J., Fucini, H. L., Dean, D. A., McPherson, D. L. & Friedman, D. A. *Proc. Natn. Acad. Sci. U.S.A.* 88, 3324-3328 (1991).
6. Buxton, K. A., Whitlady, M. N. & Yang, N. S. *Mol. Phys.* 65, 1103-1109 (1991).
7. Hofmann, C., Lüthy, P., Bultter, R. & Pliska, V. *Eur. J. Biochem.* 173, 85-91 (1988).
8. Hofmann, C. et al. *Proc. Natn. Acad. Sci. U.S.A.* 85, 7844-7848 (1988).
9. Van Rie, J., Janssens, S., Höfte, H., Dagnelie, D. & Van Melle, H. *Appl. Environ. Microbiol.* 56, 1378-1385 (1990).
10. Krawinkel, B. H. & Eiler, D. J. *Biochim. Biophys. Acta* 924, 509-514 (1987).
11. Endo, Y. & Nishikubo-Tsuno, J. *J. Invertebr. Path.* 36, 90-103 (1980).
12. Hodgson, T. C. & Eiler, D. J. *J. Gen. Virol.* 61, 97-106 (1980).
13. Gu, A. Z., Stuvardova, N. L. & Dean, D. H. *Proc. Natn. Acad. Sci. U.S.A.* 86, 4037-4041 (1989).
14. Wanner, W. R. & Whitlady, M. N. *J. Biol. Chem.* 264, 2825-2832 (1989).
15. Schmitt, H. E., Tomczak, K., Orszag, J. P. & Whitlady, M. N. *J. Biol. Chem.* 265, 20027-20039 (1990).
16. Nigg, A., Nigg, A. M., Langemann, C. A. & Schmitt, W. *J. Appl. Entomol.* 98, 590-606 (1983).
17. Höfte, H., Seidenberg, J., Van Melle, H. & Vaché, M. *Mol. Microbiol.* 10, 7183 (1991).
18. Li, J., Henderson, R., Carroll, J. & Eiler, D. J. *Mol. Cryst. Liq. Cryst.* 100, 543-549 (1983).
19. Carroll, J. J. & Eiler, D. J. *Biochem. J.* 261, 99-105 (1989).
20. Arnold, U. W. *Mol. Cryst. Liq. Cryst.* 115, 99-112 (1984).
21. Wang, B. C. *Mol. Cryst. Liq. Cryst.* 115, 99-112 (1984).
22. Janssens, S. G. W. *Acta Crystallogr.* A43, 124-126 (1987).
23. Janssens, S. G. W., Cornet, S. W. & Krijgsveld, A. *Acta Crystallogr.* A47, 110-119 (1991).
24. Krawinkel, B. H. *J. Mol. Cryst. Liq. Cryst.* 103, 803-816 (1984).
25. Chothan, C. & Felsenfeld, A. V. *Rev. Biochem.* 59, 1007-1030 (1990).

26. Chothan, C. *J. Mol. Biol.* 195, 1-15 (1976).
27. Buxton, K. A. & Felsenfeld, A. *Science* 226, 23-26 (1985).
28. Palmer, M. W., Pallen, F., Tucker, A. D. & Tsernoglou, D. *Nature* 337, 93-96 (1989).
29. Richardson, J. S. *Adv. Prot. Chem.* 34, 157-339 (1981).
30. Höfte, H. et al. *Eur. J. Biochem.* 161, 273-280 (1987).
31. Chothan, C. N. & Kaplan, H. *Biochemistry* 28, 12971-12977 (1989).
32. Nicholas, C. N., Altmann, W. & Ellis, D. J. *J. Biol. Chem.* 264, 5141-5147 (1989).
33. McPherson, S. C., McPherson, S. L., Perletti, R. J., Marrero, P. G. & Fuchs, R. L. *Biochem. Biophys. Res. Commun.* 170, 685-672 (1990).
34. Schmitt, H. E. & Whitlady, M. N. *J. Biol. Chem.* 260, 6272-6280 (1985).
35. Adams, M. J. et al. *J. Gen. Virol.* 66, 280-300 (1985).
36. Sakai, R., Thompson, D. V., Maroney, M. J., Dockland, R. G. & Adams, M. J. *Proc. Natn. Acad. Sci. U.S.A.* 84, 7036-7040 (1987).
37. Chongkrapornchai, W., Höfte, H., Seidenberg, J., Angewandten, C. & Vaché, M. *Eur. J. Biochem.* 173, 11-16 (1988).
38. Krawinkel, B. H., Thomas, W. E. & Eiler, D. J. *FEBS Lett.* 168, 101-202 (1984).
39. Krawinkel, B. H. & Eiler, D. J. *J. Cell Sci.* 63, 89-100 (1985).
40. Holder, M. Z. & Qier, D. I. *Biochem. J.* 248, 167-201 (1987).
41. Vaché, M., Munzmann, E., Sticker, A. & Dörflinger, W. G. *J. Biol. Chem.* 264, 6783-6788 (1989).
42. Lacey, J. H., Bily, D. & Parris, F. J. *J. Biol. Chem.* 264, 639-653 (1989).
43. Song, H. Y., Chen, F. S. & Chothan, W. A. *J. Biol. Chem.* 264, 2027-2034 (1989).
44. Altmann, W. & Ellis, D. J. *FEBS Microbiol. Lett.* 68, 97-106 (1989).
45. Mondscheidt, A. & Pilling, L. W. *J. Appl. Crystallogr.* 20, 306-315 (1987).
46. Nigg, A. *Acta Crystallogr.* A42, 140-149 (1986).
47. Krawinkel, B. H. *J. Appl. Crystallogr.* (in the press).

ACKNOWLEDGEMENTS. We thank P. R. Evans, A. G. W. Leslie and N. Hanson for advice and encouragement. N. Wason and Z. Chatter for help with the image plate system. K. Nigg and P. J. McLaughlin for help in collecting film data. T. A. Jones for advice on model building. SEPC Derivatives Laboratory and EMBL Outstation at DESY for use of synchrotron facilities and T. Wason and K. Nigg for maintaining the rotating anodes. D. IF and IC acknowledge the support of the AFRC.

APPENDIX B

Pfam HMM search

Page 1 of 3

Pfam 16.0 (Saint Louis)[Home](#) | [Analyze a sequence](#) | [Browse Pfam](#) | [Keyword search](#) | [Taxonomy search](#) | [Swisspfam](#) | [PfamAlyzer](#) | [Help](#)

There are 7 searches queued ahead of you on the Pfam compute server.
Please wait...

Starting search. Estimated time: 38 seconds (assuming all Wulfpac nodes are running). Please wait...

Pfam HMM search results, glocal+local alignments merged (Pfam_ls+Pfam_fs)

[\[Go here for an explanation of the format of the results\]](#)

Model	Seq- from	Seq- to	HMM- from	HMM- to	Score	E- value	Alignment	Description
II Endotoxin_N	70	293	1	244	457.8	1.2e-134	glocal	delta endotoxin, N-terminal domain
II Endotoxin_M	298	513	1	242	204.3	2.5e-58	glocal	delta endotoxin
II Endotoxin_C	523	663	1	155	121.1	2.6e-33	glocal	delta endotoxin

Alignments of top-scoring domains:Format for alignment of query to Seed:

```
Endotoxin_N: domain 1 of 1, from 70 to 293: score 457.8, E = 1.2e-134
      *->vqiglsivgtllgalgvfpaggflvgfyetlildllwpangpanenvw
query  70      ++++++ivg+ll+ lgv P++G +v++y++L+d+LWP++++s  +W
      AKAADIVGKLLBGLQV-PPVGPVIVSLYTQLIDILWPAGEKS---QW 112

      eaFleqVEqLIdQrIseyvrnrAiarLeGLgnsydtaviylealeeWekn
query  113  e+P+eqvE+LI+Q+I+ey+rn+A+++LeGLgn+y+  Yl aLeeWe+n
      RIFMRQVEELINQXIAEYARNKALSELEGLGNNYQ---LYLTALBEEWEEEN 159

      pnnaxareaVrtrFnildslfvnaipsFaveagysenyevllLPvYAQAA
query  160  pn+ r+ +Vr rP+ildslf++ +psF+v+ ++ e  v++L+vYA+AA
      PNGSRALRDVRNRPFILDSLFTQYMPsFRVT-NP-E---VPFLTVYAMAA 204

      NLHLlLLRDAvifGerWgltqadinstdednyYnrlleriksYtdHCvn
query  205  NLHLlLL+DA+ifGe+Wg +++++ln  nyY r+ +++++eY+dHCv+
      NLHLlLLKDASIFGEWGWSTTl'IN-----NYYDRQMKLTAESDHCVK 248
```

Pfam HMM search

Page 2 of 3

WYNTGLnnlrgtnldaesWvryNryRReMTLtVLDlVALFPnyDprl<-*
 wy tGL++l+gt+ a++Wv+YN++RReMTL VLD VALFPnyD+r+
 query 249 WYETGLAKLKGT--AKQWVDYNQFRREMTLAVLDVVALFPNYDTRT 293

align query/298 to Endotoxin_M (513) seed

Endotoxin_M: domain 1 of 1, from 298 to 513: score 204.3, E = 2.5e-58
 *->tkseqLRRiYTDpvevsg;eglsaglcrrWginnypxrltFsalEna
 tk+qLTRE+YTDp+g v+ ++ ++++ +p +F ++E++
 query 298 TKAQLTREVTDPGLGAVN-----VSSIGSWYDKAP--SPGVISS 335
 liRaPHLfdflnaltiyTnsargplnttldinyWsgHrvtsaytggsaln
 iR+PH fd++ lt yT s ++++ + i++W+Gh++++++
 query 336 VIRPPHVFDYITGLTVYTQSR--SISSARY-IRHWAGHQISYHRVSR---- 379
 niiasplyGnttntaepvvtispcftnndiYRlsatenrlagnniigln
 +++ yG++ n + ++t+ ftn diY+tls + ++l+ +g++
 query 380 GSNLQQMYGTNQN-LHSTSTFD--FTNYDIYKTLAKDAVLDD-IVYPGYT 425
 npingvtrvdFygangtnseisnntyres.krgngsgqrtidsidelPpet
 + +g + v+F++ n n+++ + +y++ +k + t+ds+ eLPpet
 query 426 YIFFGMPEVRPFMVNQLNNTKTLKYNFVSKDIAS--TRDSBLELPPET 473
 tnePiyesYSHrLshvtflranltqgggsdatrahvpvFswTHrSad<-*
 +++P+yesYSHrL+h+t++ + + g vpvFswTHrSad
 query 474 SDQPNYESYSHRLCHITSIPATCINTTGL-----VPVFSWTHRSAD 513

align query/298 to Endotoxin_M (513) seed

Endotoxin_C: domain 1 of 1, from 523 to 663: score 121.1, E = 2.6e-33
 *->ITQIPLVKaynlsegasVVGPGFTGGDilrrtsanGsfgtlrvttk
 ITQIP+VK VVGPG+TGGD+l+ + s+Gs+gtl +
 query 523 ITQIPAVKCDNLPFVPVVGPGHTGGDLLQYNRSTGSGVTFLARY 569
 linnplsqrYRIRIRYASTtnlrfivsliggttengfnfpkTmrgdnye
 ++ +YR+R+RYA+ +++++v+ +q+ pkTmn g e
 query 570 GLALEKAGKYRVRLRYATDADIVLHVN-----DAQIQMPKTMNPG---E 610
 dLtYeaFryaefatpvsfpyfagagqdltnistlgiqgfaggnqevYID
 dLt+++F+ at t+ ++ +++++l + +lg ++ s+ ++ vY+D
 query 611 DLTSKTFKVADAITT-LN---LATDSSLALKHNLGEDPNSTL-SGIVYVD 655
 rIEFIPvn<-*
 rIEFIPv+
 query 656 RIEFIPVD 663

align query/523 to Endotoxin_C (663) seed

NEW! Phylogenomic analysis of query using RIO.

Given a query sequence, Pfam domain, and species, the RIO server will order sequences

Pfam HMM search

Page 3 of 3

in the Pfam domain by orthology to the query. Many other options are available, and an annotated gene tree can be generated and viewed with [ATV](#). The button below will send your query and Pfam domain hits to the [RIO server](#).



[Home](#) | [Analyze a sequence](#) | [Browse Pfam](#) | [Keyword search](#) | [Taxonomy search](#) | [Swisspfam](#) | [PfamAlyzer](#) | [Help](#)

Comments, questions, flames? Email spfam@genetics.wustl.edu.

Endotoxin M <---> Enhancin

Home | Analyze a sequence | Browse Pfam | Keyword search | Taxonomy search | Swisspfam | PfamAlyzer | Help

Endotoxin_N



Figure 1: **1dlc** Toxin

Image from [PDBsum](#) database

Accession number: PF03945
delta endotoxin, N-terminal domain

This family contains insecticidal toxins produced by *Bacillus* species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N terminus is cleaved in all of the proteins and a C terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithelium and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description

This family contains insecticidal toxins produced by *Bacillus* species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N-terminus is cleaved in all of the proteins and a C-terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithelium and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description text from: [InterPro](#) entry [IPR005639](#).

Sequence Information

Alignment

☒ Seed (38) ☐ Full (173)

Format:

☒ Hyperlinked plain text ☐

[FASTA](#) [CLUSTAL](#) [MSA](#)

Visualize domain structures

☒ Seed (38) ☐ Full (173)

display per page.

[FASTA](#) [CLUSTAL](#) [MSA](#)

Species distribution

Tree depth:

☒ all ☐

[FASTA](#) [CLUSTAL](#) [MSA](#)

Literature References

[1]

Crystal structure of Insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 angstroms resolution.

Li J, Carroll J, Ellar DJ;
 Nature 1991;353:815-821.

[2]

***Bacillus thuringiensis* and its pesticidal crystal proteins.**

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feltelson J, Zeigler DR, Dean DH;
 Microbiol Mol Biol Rev 1998;62:775-806.

Database References

HOMSTRAD	endotoxin
PDB	1l5p 1dlc 1cly 1jl6
SCOP	1dlc (family)
INTERPRO	IPR005639

HMMER build information

	Pfam_ls [download HMM]	Pfam_fs [download HMM]
Gathering cutoff	-55.00 -55.00	10.00 10.00
Trusted cutoff	-52.10 -52.10	10.00 10.00
Noise cutoff	-73.30 -73.30	9.50 9.90
Build method of HMM	hmmbuild -F HMM_ls SEED hmmcalibrate --seed 0 HMM_ls	hmmbuild -f -F HMM_fs SEED hmmcalibrate --seed 0 HMM_fs

Pfam specific information

Author of entry	Bateman A, de Maagd R
Type definition	Domain
Alignment method of seed	
Source of seed members	Arne Elofsson

Home | [Analyze a sequence](#) | [Browse Pfam](#) | [Keyword search](#) | [Taxonomy search](#) | [Swisspfam](#) | [PfamAlyzer](#) |
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Pfam 16.0 (Saint Louis)
[Home](#) | [Analyze a sequence](#) | [Browse Pfam](#) | [Keyword search](#) | [Taxonomy search](#) | [Swisspfam](#) | [PfamAlyzer](#) | [Help](#)

[Endotoxin_C](#) <--- --> [Endotoxin_N](#)

Endotoxin_M



Figure 1: 1ciy Toxin
Insecticidal toxin:
structure and channel
formation

Image from [PDBsum](#) database

Accession number: PF00555
delta endotoxin

This family contains insecticidal toxins produced by Bacillus species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N terminus is cleaved in all of the proteins and a C terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithelium and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description

This entry contains insecticidal toxins produced by Bacillus species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N terminus is cleaved in all of the proteins and a C-terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithelium and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description text from [InterPro](#) entry [IPR001178](#)

Sequence Information

Alignment	Visualize domain structures	Species distribution
<input checked="" type="radio"/> Seed (38) <input type="radio"/> Full (140)	<input checked="" type="radio"/> Seed (38) <input type="radio"/> Full (140)	Tree depth:
Format:		

Hyperlinked plain text display 10 per page. all

Literature References

- [1]
Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 angstroms resolution.
 Li J, Carroll J, Ellar DJ;
 Nature 1991;353:815-821.
- [2]
***Bacillus thuringiensis* and its pesticidal crystal proteins.**
 Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feltelson J, Zeigler DR, Dean DH;
 Microbiol Mol Biol Rev 1998;62:775-806.

Database References

HOMSTRAD	endotoxin
PDB	1clx 1dlc 1j16
PfamB	PB054837
<i>The following Pfam-B family may contain sequences that according to Prodom are members of this Pfam-A family.</i>	
SCOP	1dlc (family)
INTERPRO	IPR001178

HMMER build information

	Pfam_ls [download HMM]	Pfam_fs [download HMM]
Gathering cutoff	-30.00 -30.00	10.00 10.00
Trusted cutoff	-26.00 -26.00	10.60 10.60
Noise cutoff	-36.80 -36.80	8.80 9.40
Build method of HMM	hmmbuild -F HMM_ls SEED hmmcalibrate --seed 0 HMM_ls	hmmbuild -f -F HMM_fs SEED hmmcalibrate --seed 0 HMM_fs

Pfam specific information

Author of entry	Bateman A, de Maagd R
Type definition	Domain

Alignment method of seed

Source of seed members

Arne Elofsson

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[Help](#)
Comments, questions, flames? Email spfam@genetics.wustl.edu.

Pfam 16.0 (Saint Louis)

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[Endothellin <-- --> Endotoxin_M](#)

Endotoxin_C



Figure 1: 1c1y Toxin
 Insecticidal toxin:
 structure and channel
 formation

Image from [PDBsum](#) database

Accession number: PF03944
delta endotoxin

This family contains insecticidal toxins produced by *Bacillus* species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N terminus is cleaved in all of the proteins and a C terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithellum and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description

This family contains insecticidal toxins produced by *Bacillus* species of bacteria. During spore formation the bacteria produce crystals of this protein. When an insect ingests these proteins they are activated by proteolytic cleavage. The N-terminus is cleaved in all of the proteins and a C-terminal extension is cleaved in some members. Once activated the endotoxin binds to the gut epithellum and causes cell lysis leading to death. This activated region of the delta endotoxin is composed of three structural domains. The N-terminal helical domain is involved in membrane insertion and pore formation. The second and third domains are involved in receptor binding.

Description text from InterPro entry [IPR005638](#)

Sequence Information

Alignment

☒ Seed (38) ☐ Full (159)

Format:

☒ Hyperlinked plain text ☐

☒ ClustalW alignment ☐

Visualize domain structures

☒ Seed (38) ☐ Full (159)

display per page.

☒ Pfam domain structure ☐

Species distribution

Tree depth:

☒ all ☐

☒ View species tree ☐

Literature References

[1]

Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 angstroms resolution.

Li J, Carroll J, Ellar DJ;

Nature 1991;353:815-821.

[2]

***Bacillus thuringiensis* and its pesticidal crystal proteins.**

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feltelson J, Zeigler DR, Dean DH;

Microbiol Mol Biol Rev 1998;62:775-806.

Database References

HOMSTRAD

endotoxin

PDB

1I5p 1clj 1dlc 1j16

SCOP

1dlc (family)

INTERPRO

IPR005638

HMMER build Information

	<u>Pfam_ls [download HMM]</u>	<u>Pfam_fs [download HMM]</u>
Gathering cutoff	-35.00 -35.00	13.00 13.00
Trusted cutoff	-34.80 -34.80	13.50 13.50
Noise cutoff	-36.10 -36.10	9.40 9.40
Build method of HMM	hmmbuild -F HMM_ls SEED hmmcalibrate --seed 0 HMM_ls	hmmbuild -f -F HMM_fs SEED hmmcalibrate --seed 0 HMM_fs

Pfam specific Information

Author of entry	Bateman A, de Maagd R
Type definition	Domain
Alignment method of seed	
Source of seed members	Arne Elofsson

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Comments, questions, flames? Email spfam@genetics.wustl.edu.

APPENDIX C

Attorney Docket No. 035718/237005

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re:	Abad <i>et al.</i>	Confirmation No.:	5409
Appl. No.:	10/032,717	Group Art Unit:	1638
Filed:	10/23/2001	Examiner:	A.R. Kubelik
For:	GENES ENCODING NOVEL BACILLUS THURINGIENSIS PROTEINS WITH PESTICIDAL ACTIVITY AGAINST COLEOPTERANS		

January 18, 2005

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RULE 132 DECLARATION
of
André Abad

Sir:

I, André Abad, do hereby declare and say as follows:

I am skilled in the art of the field of the invention of the above-referenced application. I earned the following academic degrees: BS majoring in mathematics and biochemistry from the Wisconsin University River Falls in 1978 and Ph.D. from Purdue University Department of Agronomy in 1996. My thesis investigated the role of a mitochondrial gene in cytoplasm male sterility in beans. From 1979 to 1991, I was employed by the University of Minnesota, Department of Plant Pathology. Working in Dr. Blanchette's laboratory, we investigated and published numerous manuscripts in the area of woody tissue degradation by fungi, in particular related to the degradation of cell wall component by fungal enzymes such as xylanase. From 1996 to 1998, I worked in Dr. Judy Bond's laboratory at Hershey Medical Center in Pennsylvania. I was involved in characterization of mouse meprin receptors and generated the constructs and ES cells necessary for producing transgenic mice targeting the knockout of meprin. Since 1999, Pioneer Hi-Bred has employed me. My current responsibility as a research scientist is to

RTA01/2173263v1

lead a research team for insecticidal protein optimization and genomic screening of *Bacillus thuringiensis* DNA for novel insecticidal genes

1. I am familiar with the experiments described in the above-mentioned application. Particularly, the procedures described in Examples 4, 6, and 7 of the above-mentioned application are considered "routine" by scientists who are familiar with research on endotoxins. Moreover, the production of plants expressing proteins having pesticidal activity, while it is a time-consuming and laborious task, is also considered "routine" by scientists who are responsible for producing such plants.

2. As one of skill in the art, given the disclosure in the above-referenced application, I would be able to make and use the claimed invention. For example, I would be able to make and use the nucleic acid of claim 1 by generating a collection of nucleic acids comprising a nucleotide sequence meeting the sequence limitation of the claims (i.e., a nucleotide sequence having at least 90% sequence identity to the nucleotide sequence set forth in SEQ ID NO:1) and assaying the encoded polypeptide for defensive activity as described in the specification, for example, as described in any of Examples 4, 6, and 7. Further, where such a collection included dozens of such sequences, I would consider this degree of experimentation to be routine rather than to be "undue experimentation." For these reasons, I believe that the claims, including for example, claim 1, are fully enabled and described by the specification.

3. It is my understanding, as one of skill in the art, that proteins can be produced that share a relatively low degree of sequence identity—maybe even as low as 70% sequence identity—with a known protein but that have the same or essentially the same function.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 01-19-2005

By: Andre A. B.

André Abad